Exhibit 91

Disruption of Iron Homeostasis in Mesothelial Cells after Talc Pleurodesis

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The mechanism for biological effects after exposure to particles is incompletely understood. One postulate proposed to explain bio logical effects after exposure to particles involves altered iron homeostasis in the host. The fibro inflammatory properties of mineral oxide particles are exploited therapeutically with the instil lation of massive quantities of talc into the pleural space, to provide sclerosis. We tested the postulates that (*1*) *in vitro* exposure to talc induces a disruption in iron homeostasis, oxidative stress, and a biological effect, and (*2*) talc pleurodesis in humans alters iron homeostasis. *In vitro* exposures of both mesothelial and airway epithelial cells to 100 μg/ml talc significantly increased iron impor tation and concentrations of the storage protein ferritin. Using dichlorodihydrofluorescein, exposure to talc was associated with a time dependent and concentration dependent generation of oxi dants in both cell types. The expression of proinflammatory media tors was also increased after *in vitro* exposures of mesothelial and airway epithelial cells to talc. Relative to control lung tissue, lung tissue from patients treated with sclerodesis demonstrated an accu mulation of iron and increased expression of iron related proteins, including ferritin, the importer divalent metal transport 1 and the exporter ferroportin 1. Talc was also observed to translocate to the parenchyma, and changes in iron homeostasis were focally distrib uted to sites of retention. We conclude that exposure to talc disrupts iron homeostasis, is associated with oxidative stress, and results in a biological effect (i.e., a fibro inflammatory response). Talc pleu rodesis can function as a model of the human response to mineral oxide particle exposure, albeit a massive one.

Keywords: talc; iron; ferritin; pleurodesis; particulate matter

Humans are routinely exposed to the particulate matter (PM) in cluded in air pollution, cigarette smoke, environmental tobacco smoke, forest fires, gas and wood stoves, and the burning of biomass other than wood, as well as those particles contacted during the min ing and processing of coal and mineral oxides. Many of the major global causes of death reported by the World Health Organization (http://www.who.int/mediacentre/factsheets/fs310/en/index.html) are related to particle exposure, and contact with PM will in crease human morbidity and mortality. This particle related mor bidity and mortality include lower respiratory infections, chronic obstructive pulmonary disease, respiratory cancers, coronary heart disease, stroke, and other cerebrovascular diseases (1 3).

CLINICAL RELEVANCE

This research concerns the disruption in iron homeostasis that occurs in the pleura and lungs of patients treated with talc pleurodesis. The accumulation of this metal, the ac companying oxidative stress, and inflammatory events after exposure to talc are comparable to those with other forms of particulate matter. Pleurodesis can function as a model of particle related biological effect.

The production of reactive oxygen species (ROS) is fundamen tal to the biological effect of PM (4), but the specific mechanism of both the generation of oxidants and its relationship with the biological effect after exposure to particles is incompletely un derstood. One postulate to explain the biological effect after exposure to particles involves altered iron homeostasis in the host after exposure. Exposure to particles introduces a solid liquid interface into cells. Oxygen containing functional groups on the PM surface provide the capacity to complex cations, and as a re sult of its high affinity for oxygen donor ligands, iron is frequently preferred. Such functional groups can include alcohols, alde hydes, and carboxylates on incompletely combusted carbon (e.g., cigarette smoke, diesel exhaust, and ambient air pollution particles), and silanol groups on silica and silicates. Particles re tained in the lung consistently demonstrate a capacity to disrupt iron homeostasis and accumulate host metal (5). Endpoints reflecting oxidative stress and a biological effect can be corre lated with this accumulation of iron that follows *in vivo* expo sure to particles (6).

Pathologic processes observed in lungs among individuals ex posed to particles include both inflammation and fibrosis (4, 7). In patients with recurrent pleural effusions, the fibro inflammatory properties of mineral oxide particles are exploited therapeutically with the instillation of massive quantities (i.e., grams) of talc into the pleural space to provide sclerosis (8). This therapeutic use affords an opportunity to examine human tissue for evidence of a disruption in iron homeostasis after an extreme exposure to mineral oxide particles. We accordingly tested the postulates that (*1*) *in vitro* exposure to talc induces a disruption of iron homeo stasis, oxidative stress, and a biological effect, and (*2*) talc pleu rodesis in humans is also associated with altered iron homeostasis and an accumulation of metal.

MATERIALS AND METHODS

Iron Uptake in an Acellular Environment

Talc (Sclerosol, Bryan Corp., Woburn, MA) was agitated in either H_2O or 1,000 μM ferric ammonium citrate for 1 hour, centrifuged, and washed with H_2O (designated talc and talc Fe, respectively). Ionizable metal concentrations associated with talc and talc Fe were measured

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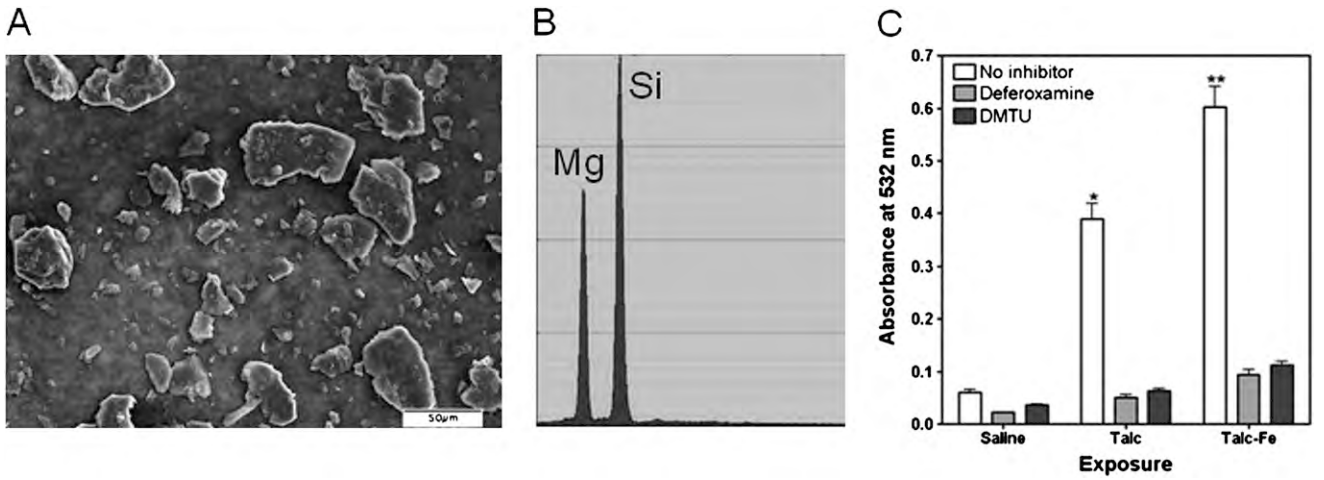

Figure 1. Particle size, composition, and capacity for generation of oxidants. (*A*) Talc was examined in a JEOL (Peabody, MA) scanning electron microscope at a screening magnification of approximately ×1,000 and a diameter ranging between 10 and 50 μm. (*B*) Energy dispersive X ray spectroscopy confirmed two peaks consistent with magnesium and silicon. (*C*). Talc demonstrated a capacity to generate hydroxyl radical in an acellular environment. This generation of oxidants was increased with a further complexation of surface iron by the talc, and was inhibited by inclusion of the metal chelator deferoxamine or the hydroxyl radical scavenger dimethylthiourea (DMTU) during incubation. *Significantly increased relative to saline. **Significantly increased relative to both saline and talc.

using inductively coupled plasma optical emission spectroscopy (ICPOES; Model Optima 4300DV; Perkin Elmer, Norwalk, CT).

Generation of Acellular Oxidants

The generation of acellular oxidants by talc and talc Fe particles was mea sured with the thiobarbituric acid reactive products of deoxyribose (9).

Cell Culture

Mesothelial cells (MeT 5A; American Type Culture Collection, Manassas, VA) were cultured in complete growth medium. In addition, BEAS 2B cells on uncoated, plastic 12 well plates in keratinocyte growth medium (Lonza, Walkersville, MD) were used.

RT PCR

Mesothelial and BEAS 2B cells were exposed to either medium alone or 100 μg/ml talc. Quantitative PCR was performed using Taqman polymerase, with detection on an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA).

Cell Iron Homeostasis

Mesothelial and BEAS 2B cells were exposed for 4 hours to 200 μM ferric ammonium citrate (FAC), 100 μg/ml talc, and both FAC and talc. Cells were then scraped into 1.0 ml 3 N HCl/10% trichloroacetic acid. After hydrolysis, concentrations of iron and zinc were determined using ICPOES. Cell incubations were repeated for 24 hours, scraped into 0.5 ml PBS, and disrupted, and ferritin concentrations in the lysates were measured using an enzyme immunoassay (Microgenics Corp., Concord, CA).

Generation of Cellular Oxidants

Mesothelial and BEAS 2B cells were grown to confluence in 96 well, white walled, tissue culture treated plates (CoStar, Lowell, MA). We loaded 2′7′ dichlorodihydrofluorescein (DCF; 20 μM) diacetate (Sigma

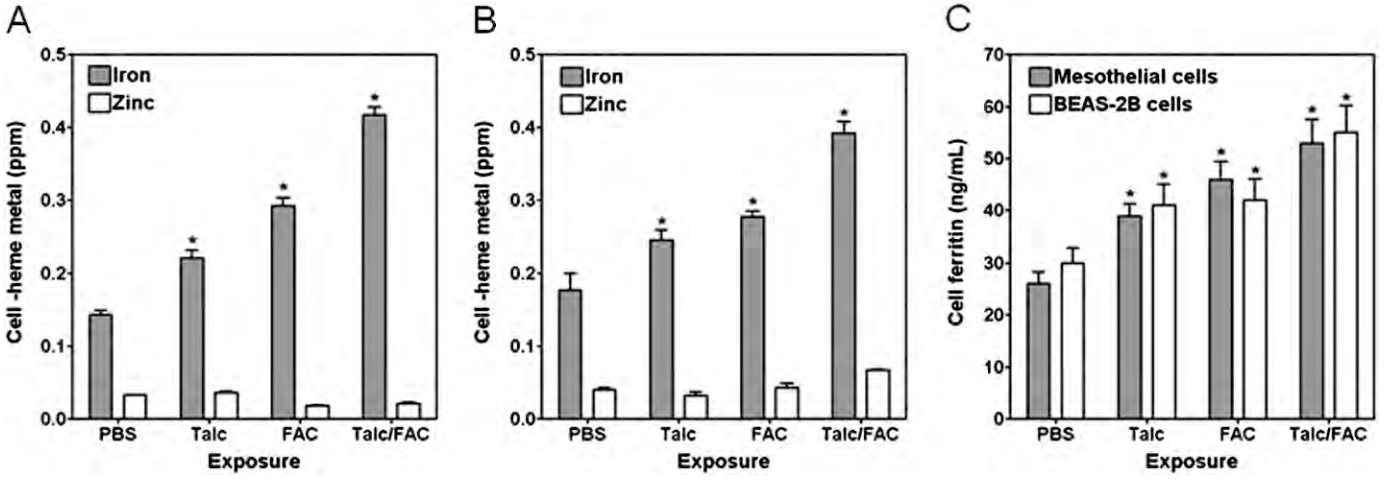

Figure 2. Cellular concentrations of nonheme metal and ferritin after *in vitro* exposure to talc. Mesothelial (*A*) and BEAS 2B cells (*B*) both increased cell nonheme iron after exposure to 100 μg/ml talc and 200 μM iron. The co incubation of cells with both talc and iron further elevated nonheme iron. Cell zinc concentration showed no change after exposures to talc and iron. (*C*) Corresponding to concentrations of nonheme iron, concentrations of cell ferritin increased in both cell types after exposures to talc and iron. *Significantly increased, relative to PBS. FAC, ferric ammonium citrate.

Case 3:16-md-02738-MAS-RLS Document 9899-1 Filed 05/30/19 Page 4 of 218 PageID: 75075

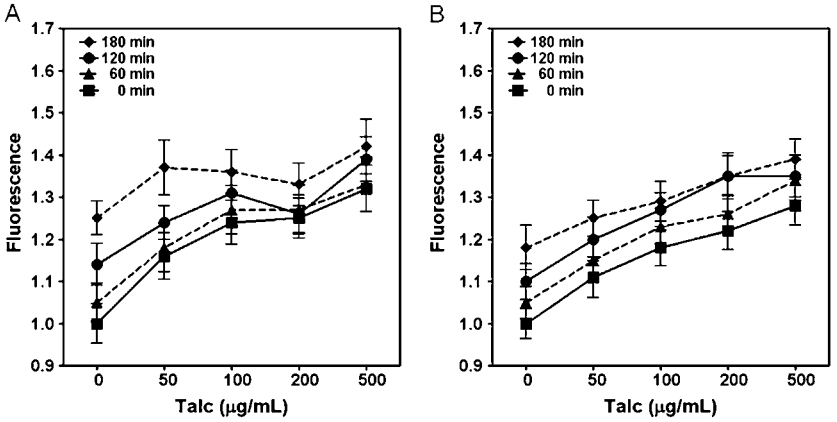

Figure 3. Cellular generation of oxidants after *in vitro* exposure to talc. Using 2′7′ dichlorodihydrofluorescein (DCF) diacetate fluorescence, mesothelial cells (*A*) and BEAS 2B cells (*B*) demonstrated a time dependent and concentration dependent generation of oxidants.

Chemical Co., St. Louis, MO), baseline readings were taken on a Perkin Elmer HTS 7000 fluorimeter using 485 nm excitation/535 nm emission filters, and PBS with and without talc was added. Fluorescence was measured at 0, 60, 120, and 180 minutes after addition.

Cellular Release of IL 8 and IL 6

Cells were exposed to either medium or 100 μg/ml talc in medium for 24 hours. Concentrations of IL 8 and IL 6 in the cell medium were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

Histochemistry and Immunohistochemisty

Our protocol was approved by the Institutional Review Board of the Duke University Health System. Staining was performed on six surgical specimens collected from patients who (*1*) had manifested talc pleurod esis 2 15 months previously (mean ± SD, 7 ± 6 months), followed by (*2*) an extrapleural pneumonectomy. Sections were stained for iron, using Perl's Prussian blue (Sigma). Immunohistochemistry for ferritin was performed using an human anti ferritin antibody (Dako, Carpinteria, CA) at a dilution of 1:100 (10) and antibodies to divalent metal transport 1 (DMT1) and ferroportin 1 (FPN1) at a dilution of 1:200 (11, 12). Control lung tissue was obtained from (*1*) patients who had undergone a pneumonectomy for lung cancer, and (*2*) individuals diagnosed with idiopathic pulmonary fibrosis (IPF) and undergoing an autopsy.

Statistical Analysis

Data are expressed as mean values ± standard errors, unless specified otherwise. Differences between multiple groups were compared using ANOVA.

RESULTS

Scanning electron microscopy of the talc showed significant variabil ity in PM size, with diameters of individual particles ranging from less than 10 μm to greater than 50 μm (Figure 1A). However, the majority of particles had diameters between 10 and 50 μm. No fibers were evident in the talc sample. Energy dispersive X ray spectroscopy revealed two peaks consistent with magnesium and silicon (Figure 1B). This finding is in agreement with the ideal molecular formula of talc ($Mg_3Si_4O_{10}(OH)_2$).

Comparable to numerous silicates (13), talc demonstrated a capacity for iron uptake in an acellular environment. Concen trations of ionizable iron were measured at 0.46 ± 0.11 ppm and 6.48 ± 1.08 ppm, respectively, for talc and talc Fe, whereas concentrations of ionizable zinc were measured at 0.03 ± 0.01 and 0.01 ± 0.00 ppm, respectively. The capacity of particles to support the *in vitro* generation of oxidants in an acellular envi ronment was significantly affected by the concentration of asso ciated iron, with talc Fe producing a significantly greater signal for malondialdehyde relative to talc (Figure 1C). This generation of oxidants was inhibited by the inclusion of either 1,000 μM deferoxamine, a metal chelator, or 1,000 μM dimethylthiourea, a hydroxyl radical scavenger (Figure 1C).

The *in vitro* exposure of mesothelial cells to talc for 4 hours resulted in an accumulation of nonheme iron. Cell zinc concen trations did not change (Figure 2A). The exposure to FAC alone confirmed the ability of mesothelial cells to import iron (Figure 2A). Co incubation with both talc and FAC was asso ciated with a greater accumulation of iron relative to FAC

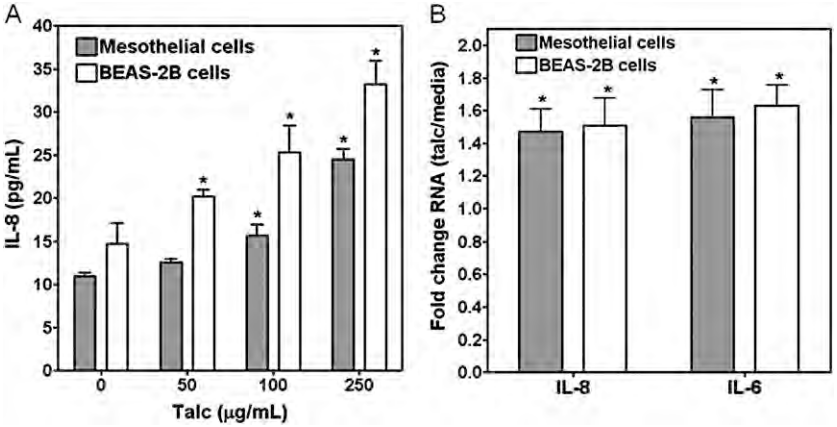

Figure 4. Release of IL 8 and changes in RNA for IL 8 and IL 6 after *in vitro* exposure to talc. (*A*) Concentra tions of IL 8 were increased after exposure to 100 μg/ml talc in both cell types. (*B*) Changes in RNA for IL 8 and IL 6 were increased 4 hours after exposure to 100 μg/ml talc. * significantly increased relative to PBS (*A*) and media (*B*).

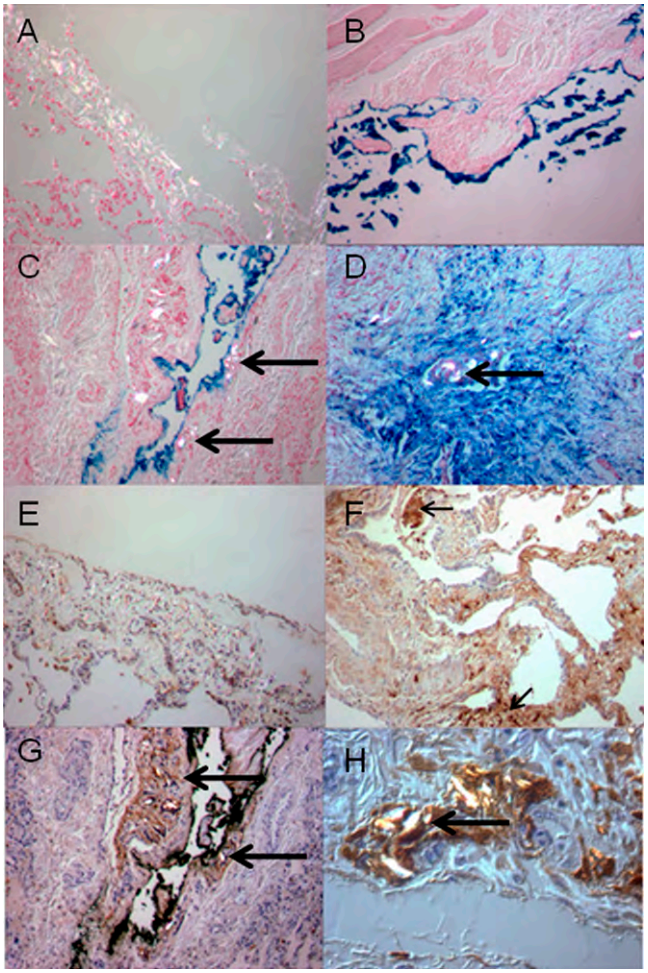

Figure 5. Stains for iron and ferritin in resected lung tissues. (*A*) Pleura from control lung tissue demonstrated no staining for iron by the pleura. Those tissue samples acquired after pleurodesis all showed positive iron staining. This staining included the parietal pleura (*B*) and visceral pleura (*C*). With translocation of the talc to both the subpleural region (*C*; *arrows* designate talc particles) and the parenchyma (*D*; *arrow* designates talc particle) of the lung, positive iron staining was evident in cells adjacent to the particle (*D*). (*E*) A small amount of uptake for the ferritin antibody by the pleura occurred in samples resected from patients treated with pneumonectomy. Specifically, this staining localized to the single cell layer of mesothelial cells and the loose fibrous tissue immediately beneath it. (*F*) Some expression of ferritin was also evident in chronic inflammatory cells resident in the lungs of patients with idiopathic pulmonary fibrosis (IPF; *arrows* designate areas of chronic inflammation distorting the alveolar and septal regions). (*G*) However, in patients with pleurodesis, talc was evident in the subpleural region, and uptake of the ferritin antibody by the pleura was very strong (*arrows* designate particles). (*H*) Talc was also detected in the lung parenchyma, and ferritin staining was similarly increased in cells adjacent to the particle (*arrow* designates particle). The expression of ferritin in the lungs of patients with pleurodesis was focally distributed to sites of talc retention. *Magnification*, approximately ×100.

exposure alone (Figure 2A). BEAS 2B cells exposed to talc and FAC revealed similar increases in cell nonheme metal. Again, co incubations revealed an increased cell iron import relative to either talc or FAC alone (Figure 2B). These results demonstrate that the capacity of talc to complex iron in an acellular environment is retained during *in vitro* mesothelial and airway epithelial cell exposures. In addition, these data suggest that talc could deplete intracellular sources of iron, and resulting in the importation of greater quantities of metal. Comparable to cell nonheme iron concentration, an increase in cell ferritin occurred after exposures of mesothelial and airway epithelial cells to talc, and this increase was even greater after exposures to both FAC and talc (Figure 2C). RNA for ferritin, DMT1 (a major iron importer), and FPN1 (a major iron exporter) did not significantly change 4 hours after exposure of mesothelial cells to 100 μg/ml talc (Table E1 in the online supplement).

A fluorescence method using DCF diacetate demonstrated an increased generation of ROS by both mesothelial and BEAS 2B cells after exposure to talc (Figures 3A and 3B). This cellular generation of oxidants after exposure to particles was both time dependent and concentration dependent, supporting a capacity of cells to produce ROS after exposure to talc. RNA for heme oxygenase and cyclooxygenase, which are potential intracellular sources of oxidative stress, showed conflicting responses, with heme oxygenase significantly increasing in both mesothelial and airway epithelial cells 4 hours after exposure to 100 μg/ml talc, and the concentration of cyclooxygenase not changing (Table E1). Finally, mesothelial and BEAS 2B cells both increased the release of IL 8 after exposure to 100 μg/ml talc (Figure 4A). IL 6 demonstrated a trend toward increasing after exposure to talc, but significant differences were not evident. RNA for IL 8 and IL 6 similarly increased after a 4 hour exposure to talc in both mesothelial cells and BEAS 2B cells (Figure 4B). This increased release of proinflammatory mediators reflects a relevant *in vitro* biological response in both cell types after talc exposure.

All patients with pleurodesis were male. Their mean age (± SD) was 56 ± 18 years. All but one manifested malignant mesothelioma (the exception underwent surgical intervention for repeated pneumothoraces). Tissue specimens, used as control samples, were collected at the time of pneumonectomy for lung cancer ($n = 6$) and at autopsy of patients with IPF ($n = 6$). These individuals manifested no pleurodesis. The specimens obtained during pneumonectomy reflected tissue uninvolved by fibrosis, whereas specimens from patients diagnosed with IPF included fibrotic specimens unrelated to mineral oxide exposure. Control tissue collected during pneumonectomy and autopsy were from men with mean ages (± SD) of 70 ± 15 years and 68 ± 9 years, respectively. Control lungs demonstrated no staining for iron in the pleura or parenchyma (Figure 5A). Significant iron staining occurred in all specimens collected after pleurodesis. Iron was localized to both the parietal (Figure 5B) and visceral (Figure 5C) pleura. Some of the talc was observed to be subpleural (Figure 5C), and particles were also evident in the parenchyma of the lungs (Figure 5D). When particles moved subpleurally and into the parenchyma, they retained their capacity to accumulate the metal (Figure 5D). Some uptake of the ferritin antibody by pleura occurred in the control specimens collected from patients with lung cancer (Figure 5E) and IPF, but this uptake was minimal. Among patients with IPF, uptake for the ferritin body corresponded to areas of inflammation (Figure 5F). After exposure to talc, the staining for ferritin was so intense that the visceral pleura appeared black (Figure 5G). After transport to the lung parenchyma, talc continued to demonstrate an association with an increased expression of ferritin protein (Figure 5H). The expression of this storage protein was focally distributed to the talc, with increased staining immediately adjacent to the particle.

Little staining for DMT1 was evident in the pleura of lungs resected from control patients, and this staining was limited to respiratory epithelia (Figures 6A and 6B). The expression of this major iron importer in the pleura increased enormously after pleurodesis with talc (Figure 6C). Uptake for the iron

Case 3:16-md-02738-MAS-RLS Document 9899-1 Filed 05/30/19 Page 6 of 218 PageID: 75077

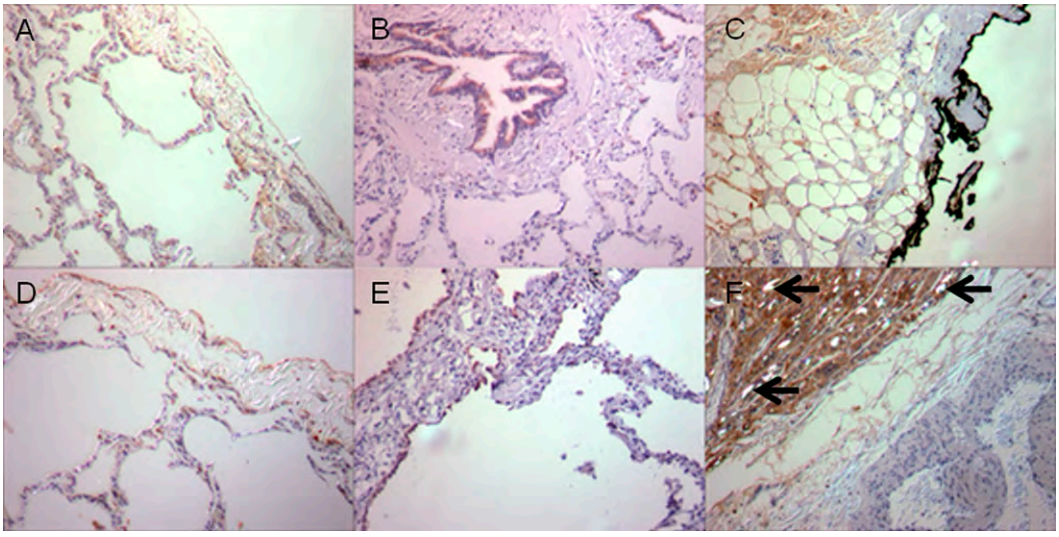

Figure 6. Divalent metal transport 1 (DMT1) and ferroportin 1 (FPN1) staining in resected lung tissue. Some binding of the antibody to both DMT1 and FPN1 was evident in lung tissue resected from patients unexposed to talc (*A* and *D*, respectively). Similarly, the uptake of antibodies for DMT1 and FPN1 in autopsy samples from patients with IPF was low (*B* and *E*, respectively). However, the expression of the iron importer DMT1 and iron exporter FPN1 both increased significantly among patients treated with pleurodesis (*C* and *F*, respectively). Comparable to the expression of ferritin, increased uptake for the antibodies to DMT1 and FPN1 occurred after translocation of the particle to the lung parenchyma (*F*; *arrows* designate particles). *Magnification*, approximately ×100.

exporter FPN1 was negligible among lungs resected from control patients (Figures 6D and 6E). Among those patients with talc pleurodesis, the expression of FPN1 was greatly increased at the pleura and in the lung cells immediately adjacent to translocated particles (Figure 6F). Finally, trichrome staining for collagen demonstrated minuscule, subpleural staining in the control specimens taken during pneumonectomy for lung cancer (Figure 7A). In contrast, sheets of collagen were verified by trichrome staining in lung tissue resected after talc pleurodesis (Figure 7B). In the lungs of patients with pleurodesis, the talc appeared at the periphery of the fibrosis (Figure 7B).

DISCUSSION

The surfaces of silica and silicate particles, including talc, contain some concentration of silanol groups (Si OH). Si^{4+} has a high electron affinity, and the Si O bond consequently has a significant ionic character and an acidic dissociation constant favoring dissociation at physiologic pH (14). The dissociation of silanol groups contributes to a net negative charge on the particle surface, which generates a capacity for the adsorption and exchange of cations (15). The open network of negatively charged silanol groups on a silica and silicate surface presents spaces large enough to accommodate adsorbed metal cations. As a result of its electropositivity, Fe^{3+} has a high affinity for oxygen donor ligands (16, 17), and reacts with the silanol group to form a silicato iron coordination complex (18):

$$Fe^{3+} + m(\quad SiOH) \rightarrow Fe(OSi)_m^{3\ m+} + mH^+.$$

The dose dependent adsorption of inorganic iron was demonstrated for surface silanol groups on crystalline silicates, with critical stability constants up to $1 \times 10^{17.15}$ (critical stability constant = 17.15) (19, 20). Our investigation confirmed the capacity of talc to complex iron from an acellular source comparable to other silica and silicate particles (13). The *in vitro* exposure to talc similarly affected an accumulation of nonheme iron in both mesothelial and airway epithelial cells. Although the source of this accumulated iron was not identified, the talc surface may complex host metal originally associated with ATP, ADP, GTP, citrate, DNA, free amino acids (21), and mitochondrial sources. However, the cell will recognize this loss of requisite iron, and subsequently increases metal import. After integration of the particle's capacity for complexation into the cell's requirement for iron, increased metal uptake and storage would occur. Reflecting these processes, concentrations of ferritin increased after *in vitro* exposure to talc. As a result of posttranscriptional control acting as a major contributor to expression (22 24), the

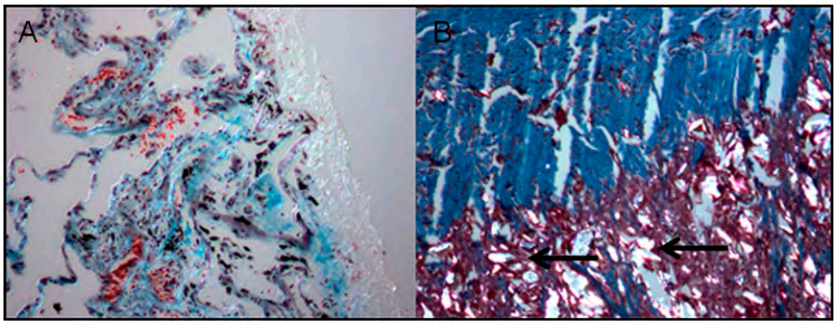

Figure 7. Masson's trichrome stain for collagen in resected lung tissue. (*A*) Control lungs demonstrated little trichrome staining (*blue*). (*B*) In contrast, tissue collected from patients with pleurodesis showed dense sheets of collagen. Talc was evident at the periphery of this collagen deposition, comparable to a silica nodule (*arrows* designate particles). *Magnification*, approximately ×100.

RNA for ferritin, DMT1, and FPN1 did not change in mesothelial and BEAS 2B cells.

Comparable to *in vitro* accumulation of iron after exposure to talc, significant metal accumulation occurred *in vivo* after pleurodesis. Perl's Prussian blue staining demonstrated metal in close proximity to retained talc particles in both the mesothelial cells of the pleura and the macrophages and airway epithelial cells of the parenchyma. Because the iron originally associated with the talc approximated normal cell and tissue concentrations, the accumulation of metal after pleurodesis had to originate from host sources of iron. Immunohistochemistry for ferritin showed elevations in expression by mesothelial cells at the pleura and in parenchymal cells after the introduction of talc. The stain for ferritin suggested a very focal response, with cells either directly contacting or immediately adjacent to the particle demonstrating uptake of the antibody to this storage protein. Similarly, the expression of the iron importer DMT1 and the iron exporter FPN1 both increased on staining in those patients treated with talc pleurodesis. In control tissue from patients with lung cancer and IPF, no positive staining for iron was evident. The uptake of the antibody to ferritin, DMT1, and FPN1 in these same tissues was minimal, but was evident in mesothelial cells of the visceral pleura, airway epithelial cells, and alveolar macrophages. In addition, ferritin was evident in the chronic inflammatory cells of autopsy samples from patients with IPF. The evidence for disrupted iron homeostasis in IPF was not compelling and this pathway is not proposed as a mechanism of biological effect in all fibrotic injuries.

Elevations in tissue concentrations of ferritin, DMT1, and FPN1 in tissues collected after pleurodesis challenge current understanding, because the controls of expression can be diametrically opposite to each other (22). The expression of these three proteins involves the same posttranscriptional mechanism, using the iron responsive element (IRE). For ferritin and FPN1, a specific sequence at the 5′ untranslated end of ferritin mRNA (i.e., the IRE) binds a cubane iron sulfur cluster, referred to as the iron regulatory protein (IRP), when the IRP exists in the apoprotein form. Elevated concentrations of available iron react with IRP to alter its conformation, decrease affinity of the protein to the mRNA, and displace it from the mRNA, allowing translation to proceed. In contrast, DMT1 mRNA contains an IRE at the 3′ untranslated region that allows increased synthesis with iron depletion. The elevation in expression of all three of these proteins after pleurodesis supports an iron depletion that occurs after the complexation of essential cell metal by the surface of the endocytosed talc particle. This initiates an increase in intracellular iron as the cell elevates concentrations to meet the demands of a new equilibrium imposed by the talc. Subsequently, all three proteins can be elevated, but such expression may involve temporal and regional variability.

The disruption of cell iron homeostasis is frequently associated with oxidative stress (25, 26). In a similar manner, *in vitro* exposure to talc increased the generation of oxidants, measured as DCF diacetate fluorescence, by mesothelial and airway epithelial cells. The acellular assay for hydroxyl radical production suggests that surface functional groups on the talc complex a source of available iron in the cell, which then redox cycles, generating oxidant. An alternative proposal could involve talc sequestering cell iron and the host response involving superoxide generation as a ferrireductant to resecure the requisite metal. *In vitro* changes in heme oxygenase RNA after cellular exposures to talc also support oxidative stress.

The biological effect of talc was evaluated using indices of the fibro inflammatory response. The *in vitro* cellular response to talc included the increased release of IL 8 and an elevation of RNA for both IL 8 and IL 6, comparable to those for many particles (27). Among patients treated with talc pleurodesis, the deposition of collagen was obvious on trichrome staining, again analogous to numerous particle exposures (6). The indices of both inflammation and fibrosis after exposure to mineral oxide particles were demonstrated to correlate with changes in iron homeostasis. The release of inflammatory mediators can directly correspond to the concentration of metal complexed to a particle surface (28). Regarding the relationship between iron and fibrotic injury, exposure to metal chelators such as bleomycin can increase the activity of prolyl hydroxylase, resulting in a deposition of collagen (29). *In vitro* and *in vivo* exposures both confirm that biological effects after talc include those proinflammatory and fibrotic events previously associated with mineral oxide particles.

We conclude that exposure to talc disrupts iron homeostasis in mesothelial and airway epithelial cells, and is associated with both oxidative stress and a biological effect, comparable to those of other particles. The resultant accumulation of iron and alterations in iron related proteins are evident among patients with pleurodesis. Sclerosis after exposure to talc can be regarded as a model of therapeutic benefit after a massive and focal exposure to a mineral oxide particle.

Author Disclosure: A.J.G., J.M.S., L.A.D., J.H.R., J.L.T., and E.N.P. do not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. V.L.R. has testified in cases involving talc manufacturers in asbestos litigation.

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Case 3:16-md-02738-MAS-RLS Document 9899-1 Filed 05/30/19 Page 8 of 218 PageID: 75079

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Exhibit 92

Cytotoxicity and Apoptosis Induction by Nanoscale Talc Particles from Two Different Geographical Regions in Human Lung Epithelial Cells

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ABSTRACT: We have characterized the physicochemical properties of nanotalc particles from two different geographical regions and examined their toxicity mechanisms in human lung epithelial (A549) cells. Indigenous nanotalc (IN) of Indian origin and commercial nanotalc (CN) of American origin were used in this study. Physicochemical properties of nanotalc particles were characterized by X-ray diffraction (XRD), transmission electron microscopy (TEM), energy dispersive X-ray spectroscopy (EDS), Brunauer-Emmet-Teller (BET), and dynamic light scattering (DLS). Results showed that both IN and CN particles significantly induce cytotoxicity and alteration in cell cycle phases. Both IN and CN particles were found to induce oxidative stress indicated by induction of reactive oxygen species (ROS), lipid peroxidation, and depletion of antioxidant levels. DNA fragmentation and caspase-3 enzyme activation due to IN and CN particles exposure were also observed. We further showed that after iron chelation, IN and CN particles produce significantly less cytotoxicity, oxidative stress, and genotoxicity to A549 cells as compared with nonchelated particles. In conclusion, this study demonstrated that redox active iron plays significant role in the toxicity of IN and CN particles, which may be mediated through ROS generation and oxidative stress. © 2012 Wiley Periodicals, Inc. Environ Toxicol 29: 394 406, 2014.

Keywords: nanotalc particles; physicochemical characterization; iron chelation; toxicity; apoptosis

INTRODUCTION

Talc is a mineral compound [$Mg_3Si_4O_{10}$ $(OH)_2$] with unique attributes and significant commercial importance. Talc is widely used due to its intrinsic properties such as high thermal stability, low electrical conductivity, good absorption and adsorption properties, and high crystallinity (Pérez-Maqueda et al., 2005; Nkoumbou et al., 2008). Talc is utilized in various applications including paper, paint, cosmetic, plastic, ceramic, pesticide, and pharmaceuticals (Carretero, 2002; Bizi et al., 2003; Petit et al., 2004). Hence, occupational and consumer exposures to talc particles are wide and complex (Jaynes and Zartman, 2005). It has been reported that talc mine workers show higher rates of lung cancer and other respiratory diseases (National Toxicology Program, 1993). Epidemiologic evidence also suggests a possible association between genital use of talcum powder and risk of ovarian cancer (Wild,

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2006; Buz'Zard and Lau, 2007; Gates et al., 2008; Langseth et al., 2008). Talc also appears to induce reactive oxygen species (ROS) generation, oxidative stress, and inflammation (Harlow and Hartge, 1995; Buz'Zard and Lau, 2007).

Due to enhanced intrinsic properties, nanoscale talc particles are extensively utilized in many commercial and industrial products (Akhtar et al., 2008; Balamurugan and Maiti, 2010; Sakthivel and Pitchumani, 2011). Despite the wide-spread applications, there is a serious lack of information concerning the mechanisms of toxicity of nanotalc particles. Previously, we have observed that human cells exposed to nanotalc particles induce oxidative stress-mediated cytotoxicity (Akhtar et al., 2010a). However, physicochemical characterization of nanotalc particles and their association with the toxicological response in human cells is still remains unclear.

There are numerous reports suggesting that ROS is an important mediator of the toxicity of minerals such as asbestos and silica (Aung et al., 2007; Akhtar et al., 2010b). It has been known for years that the surface iron (II) or leachable iron (II) on mineral surfaces reduces molecular oxygen to superoxide anion, which is then dismutated to hydrogen peroxide (Shukla et al., 2003). In the presence of asbestos or silica, hydrogen peroxide and superoxide react via a Fenton reaction and/or Haber Weiss reaction driven by iron to form the potent hydroxyl radical in vitro leading to cellular oxidative damage (Persson et al., 2003).

The aim of this work was to characterize the physicochemical properties of nanotalc particles and to determine the role of iron in the toxicity mechanisms of nanotalc particles in human lung epithelial (A549) cells. We utilized two types of nanotalc particles from different geographical origins; indigenous nanotalc (IN) of Indian origin and commercial nanotalc (CN) of American origin. Cytotoxicity of IN and CN particles was examined by MTT and LDH assays. Oxidative stress response of IN and CN particles was assessed by measuring reactive oxygen species (ROS), lipid peroxidation (LPO), glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT). Apoptotic response of IN and CN particles was evaluated by cell cycle analysis, DNA fragmentation, and caspase-3 enzyme activity. To explore the role of iron in the toxicity of IN and CN particles, we utilized deferoxamine mesylate (DFOM), a well-known iron chelator. The physicochemical properties of IN and CN particles were characterized by X-ray diffraction (XRD), transmission electron microscopy (TEM), energy dispersive X-ray spectroscopy (EDS), Brunauer-Emmet-Teller (BET), and dynamic light scattering (DLS). The A549 cells, derived from human lung carcinoma, have been widely utilized in toxicological studies (Zhang et al., 2010; Akhtar et al., 2010a,b; Ahamed et al., 2011a,b,c).

MATERIALS AND METHODS

Nanotalc Particles and Reagents

We have utilized the nanotalc particles from two different geographical regions. Indigenous nanotalc (IN) particles were collected from Rajasthan, India, as reported in our previous publication (Akhtar et al., 2010a). American origin commercial nanotalc (CN) particles (size 70 12 nm) were purchased from M.K. Impex (Mississauga, Canada).

Fetal bovine serum (FBS), penicillin-streptomycin, DMEM/F-12 medium, and HBSS were purchased from Invitrogen Co. (Carlsbad, CA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide], 2,7-dichlorofluorescin diacetate (DCFH-DA), deferoxamine mesylate (DFOM), glutathione (GSH), thiobarbituric acid (TBA), propidium iodide (PI), RNase A, diethylenetriaminepentaacetic acid (DETAPAC), *N*-acetyl-asp-glu-val-asp-7-amido-4-trifluoromethylcoumarin (Ac-DEVD-AFC), 7-amido-4-trifluoromethylcoumarin (AFC) standard, Bradford reagent, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO). Apoptotic DNA Ladder Kit was bought from Roche. All other chemicals used were of the highest purity available from commercial sources.

Characterization of Nanotalc Particles

Crystalline nature of both IN and CN particles were examined by taking X-ray diffraction (XRD) pattern at room temperature with the help of PANalytical X'Pert X-ray diffractometer equipped with a Ni filtered using Cu-K_α (λ = 1.54056 Å) radiations as X-ray source. Morphology and size of IN and CN particles were examined by field emission transmission electron microscopy (FETEM) (JEM-2100F, JEOL Inc., Tokyo, Japan) at an accelerating voltage of 200 kV. To check the purity of IN and CN particles, an energy dispersive X-ray spectroscopy (EDS) analysis was performed. Brunauer-Emmet-Teller (BET) surface area measurement of IN and CN particles was determined by multipoint nitrogen adsorption at 77 K using a Beckman Coulter SA3100 device.

Dynamic light scattering (DLS) and laser Doppler velocimetry (LDV) for the characterization of hydrodynamic size and zeta potential (ζ) of IN and CN particles in distilled water and cell culture media were performed on a Malvern Instruments Zetasizer Nano-ZS instrument as described by Murdock et al. (2008).

Treatment of Nanotalc Particles with Deferoxamine Mesylate

We treated both IN and CN particles with DFOM for iron chelation. In brief, IN and CN particles were incubated with 10 mM DFOM at a concentration of 1000 μg/mL for

20 h as described by Aung et al. (2007). Then particles were washed three times with cell culture medium by centrifuging at 4000 rpm for 10 min followed by resuspension.

Cell Culture and Exposure to Nanotalc Particles

Human lung epithelial (A549) cells were obtained from National Centre for Science (NCCS), Pune, India. Cells were used between passages 10 and 20. Cells were cultured in DMEM/F-12 medium supplemented with 10% FBS and 100 U/mL penicillin-streptomycin at 5% CO_2 and 37°C. At 85% confluence, cells were harvested using 0.25% trypsin and were subcultured into 75 cm^2 flasks, 6-well plates, or 96-well plates according to selection of experiments. Cells were allowed to attach the surface for 24 h before treatment. IN and CN particles were suspended in cell culture medium and diluted to a appropriate concentration (200 μg/mL). Suspension of nanotalc particles were then sonicated using a sonicator bath at room temperature for 10 min at 40 W to avoid particles agglomeration before administration to the cells. The selection of the 200 μg/mL concentration of nanotalc particles was based on our previous publication (Akhtar et al., 2010a). All the data presented in this study was that of 48 h exposure. Cells not exposed to nanotalc particles served as controls in each experiment.

Cell Viability Assay

Viability of A549 cells after exposure to nanotalc particles was assessed by MTT assay as described by Mossman (1983). The MTT assay assesses the mitochondrial function by measuring ability of viable cells to reduce MTT into blue formazon product. In brief, 10,000 cells per well were seeded in 96-well plate and exposed to IN and CN particles at the concentration of 200 μg/mL for 48 h. After the exposure completed, the medium was removed from each well to avoid interference of particles and replaced with new medium containing MTT solution in an amount equal to 10% of culture volume, and incubated for 3 h at 37°C until a purple colored formazan product developed. The resulting formazan product was dissolved in acidified isopropanol. Further, the 96-well plate was centrifuged at 2500 rpm for 5 min to settle down the remaining particles present in the solution. Then, a 100 μL supernatant was transferred to other fresh wells of 96-well plate and absorbance was measured at 570 by using a microplate reader (FLUOstar-Omega).

Lactate Dehydroganase Leakage Assay

Lactate dehydrogenase (LDH) is an enzyme widely present in cytosol that converts lactate to pyruvate. When plasma membrane integrity is disrupted, LDH leaks into culture media and its extracellular level is elevated. LDH assay was carried out with the method described earlier (Wroblewski and LaDue, 1955; Welder et al., 1991). In brief, 10,000 cells per well were seeded in 96-well plate and exposed to IN and CN particles at the concentration of 200 μg/mL for 48 h. After the exposure completed, the 96-well plate was centrifuged at 2500 rpm for 10 min to get the cell culture media. Then, a 100 μL of culture media transferred to new fresh tube containing 100 μL of sodium pyruvate (2.5 mg/mL phosphate buffer) and 100 μL of reduced nicotinamide adenine dinucleotide (NADH) (2.5 mg/mL phosphate buffer) in a total volume of 3.0 mL (0.1 M potassium phosphate buffer, pH 7.4). The rate of NADH oxidation was determined by following the decrease in absorbance at 340 nm for 3 min at 30-s interval using a spectrophotometer (Thermo-Spectronic).

Cell Cycle Analysis

Cell cycle distribution was assayed by determining DNA content. Cells were treated with IN and CN particles for 48 h. After exposure, cells were fixed in 3% (w/v) paraformaldehyde for 10 min, permeabilized on ice in phosphate buffer saline-0.5% Triton X-100 for 15 min, washed and resuspended in 0.5 ml of phosphate buffer saline containing 1% FBS, 1 mg/ml RNaseA, and 50 μg/ml propidium iodide. The samples were incubated for 30 min at 37°C. The data were acquired and analyzed on FACS-Calibur flow cytometer (Becton-Dickinson LSR II, San Jose, CA) using Cell Quest 3.3 software.

Measurement of Reactive Oxygen Species

For the measurement of ROS generation, cells were cultured in 12-well plate. The production of intracellular ROS was measured using 2,7-dichlorofluorescin diacetate (DCFH-DA) (Wang and Joseph, 1999). The DCFH-DA passively enters the cell where it reacts with ROS to form the highly fluorescent compound dichlorofluorescein (DCF). Briefly, 10 mM DCFH-DA stock solution (in methanol) was diluted in culture medium without serum or other additive to yield a 100 μM working solution. After exposure to IN and CN particles, cells were washed twice with HBSS and then incubated in 1 mL working solution of DCFH-DA at 37°C for 30 min. Cells were lysed in alkaline solution and centrifuged at 3000 rpm for 10 min. Then, a 200 μL supernatant (from 12-well plate) was transferred to the fresh well of black 96-well plates and fluorescence was measured using at 485 nm excitation and 520 nm emission using a microplate reader (FLUOstar-Omega). The values of ROS were expressed as percent of fluorescence intensity relative to controls.

Membrane Lipid Peroxidation Assay

The extent of membrane lipid peroxidation (LPO) was estimated by measuring the formation of thiobarbituric acid reactive species (TBARS) using the method of Ohkawa et al. (1979). Briefly, cells were cultured in 75 cm^2 culture flask and exposed to IN and CN particles at the concentration of 200 μg/mL for 48 h. After the treatment, a 200 μL of cell suspension was mixed with 800 μL of LPO assay cocktail containing TBA (0.4%, w/v), sodium dodecyl sulphate (0.5%, w/v), and acetic acid (5 %, v/v). Reaction mixture was then incubated at 95°C for 1 h. After cooling to room temperature the reaction mixture was centrifuged at 5000 rpm for 5 min. The absorbance of the supernatants was read at 532 nm against the standard. The amount of TBARS was expressed as nmol/mg protein.

Intracellular Glutathione Assay

Intracellular GSH was quantified using the method of Hissin and Hilf (1976). Briefly, cells were cultured in 75 cm^2 culture flask and exposed to IN and CN particles at the concentration of 200 μg/mL for 48 h. After the exposure completed, cells were lysed in 20 mM Tris (pH 7.0) and the centrifuged at 10,000 rpm for 10 min at 4°C. Further, protein of the supernatant was precipitated using 1% perchloric acid and again centrifuged at 10,000 rpm for 5 min at 4°C to get supernatant. Then 20 μL of supernatant was mixed with 160 μL of 0.1M potassium phosphate-5 mM EDTA buffer (pH 8.3) and 20 μL *O*-phthalaldehyde (1 mg/mL in methanol) in a black 96-well plate. After 2 h of incubation at room temperature in the dark, fluorescence was measured at emission wavelength of 460 nm and excitation wavelength of 350 nm. The amount of GSH was expressed as nmol GSH/mg protein.

Antioxidant Enzymes Activity Assay

Cells were cultured in 75 cm^2 culture flask and exposed to IN and CN particles at the concentration of 200 μg/mL for 48 h. After the exposure completed, cells were harvested in ice-cold phosphate buffer saline and washed twice with phosphate buffer saline at 4°C. The cell pellets were then lysed in cell lysis buffer. Following centrifugation (10,000 rpm for 10 min 4°C) the supernatant (i.e. cell lysate) was maintained on ice until assayed for activity of superoxide dismutase (SOD) and catalase (CAT) enzymes. The total SOD was determined using pyrogallol assay following the procedure described by Marklund and Marklund (1974), based on the competition between pyrogallol oxidation by superoxide radicals and superoxide dismutation by SOD, and spectrophotometrically read at 420 nm. The amount of SOD inhibiting the reaction rate by 50% in the given assay conditions was defined as one unit of SOD. The results were expressed as units/min/mg protein.

CAT activities were assayed by the method described by Claiborne (1985). One unit of CAT activity is defined as the amount of enzyme that decomposes 1 μmol H_2O_2/min. CAT activities were given as μmol H_2O_2 decomposed/min/mg protein.

DNA Ladder Assay

Cells were cultured in 6-well plate and exposed to IN and CN particles at the concentration of 200 μg/mL for 48 h. At the end of exposure DNA was extracted using an apoptotic DNA Ladder Kit (Roche, Cat# 11835246001). The extracted DNA was then evaluated on a 1% agarose gel using ethidium bromide. DNA fragmentation pattern was documented by a gel documentation system.

Assay of Caspase-3 Enzyme

Cells were cultured in 6-well plate and exposed to IN and CN particles at the concentration of 200 μg/mL for 48 h. Activity of caspase-3 enzyme was determined using a standard fluorometric microplate assay (Walsh et al., 2008) with some modifications. A reaction mixture containing 30 μL of cell lysate, 20 μL of Ac-DEVD-AFC (caspase-3 substrate), and 150 μL of protease reaction buffer (50 mM Hepes, 1 mM EDTA, and 1 mM DTT), pH 7.2, was incubated for 15 min. Fluorescence of reaction mixture was measured at 5 min interval for 15 min at excitation/emission wavelengths of 430/535 nm using a microplate reader (FLUOstar-Omega). A standard of 7-amido-4-trifluoromethylcoumarin (AFC) ranging from 5 to 15 μM was prepared and its fluorescence was recorded for calculation of caspase-3 activity in terms of pmol AFC released/min/mg protein.

Estimation of Protein

The protein content was measured by the method of Bradford (1976) using Bradford reagent (Sigma-Aldrich, St. Louis, MO), along with bovine serum albumin as standard.

Statistics

All the data represented in this study are means ± SD of three identical experiments made in three replicate. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Significance was ascribed at $p < 0.05$. All analyses were conducted using the Prism software package (GraphPad Software).

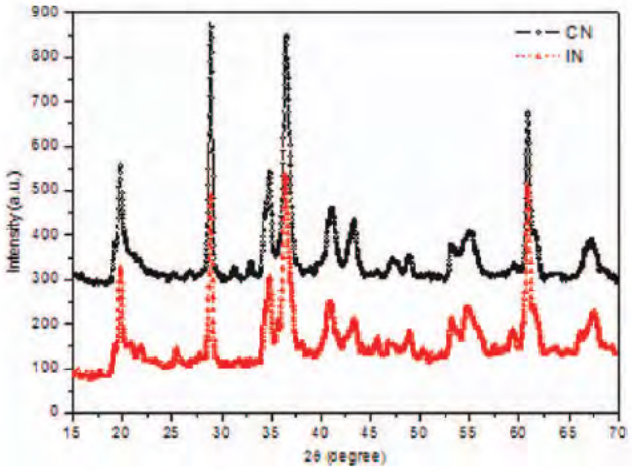

Fig. 1. XRD pattern of two types of nanotalc particles. IN; indigenous nanotalc particles, CN; commercial nanotalc particles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

RESULTS

Characterization of IN and CN Particles

Characterization of IN and CN particles was performed using a combination of XRD, TEM, DLS, zeta-potential, and BET in order to provide clear insight into crystalline nature, morphology, particle size, surface property, and chemical composition. These properties are necessary for a better understanding of nanotoxicology.

Figure 1 represents the XRD pattern of IN and CN particles. Image clearly exhibits that the crystalline nature of both IN and CN particles were same. The average size of nanocrystals calculated from the XRD results using Scherrer's equation (Patterson, 1939) was found to be 93 and 89 nm for IN and CN particles, respectively. Figure 2(A,B) show the typical TEM images of IN and CN particles, respectively. Images show that particles are aggregated. We never found small independent crystals in the TEM images.

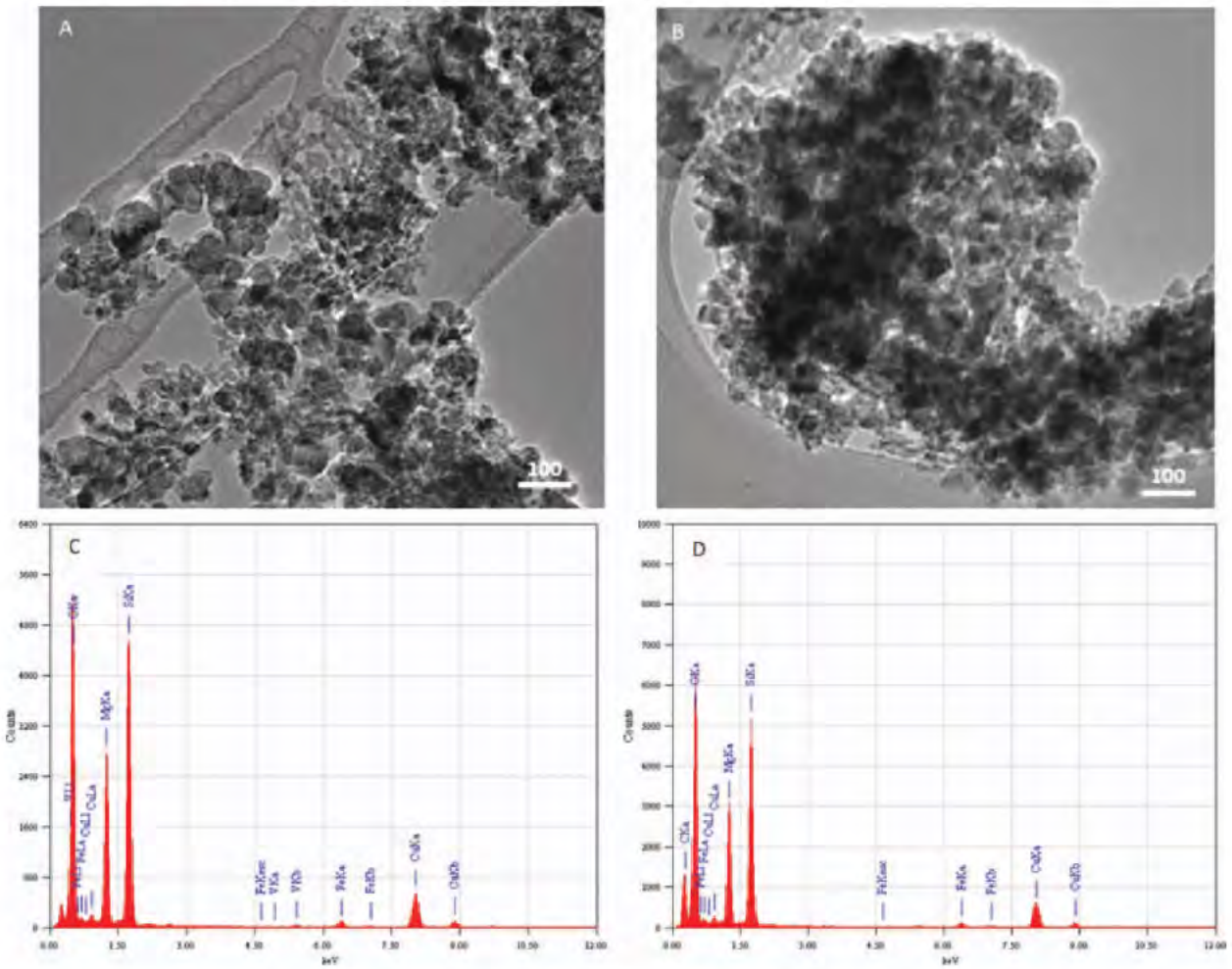

Fig. 2. TEM characterization of nanotalc particles. (A) FETEM of indigenous nanotalc particles, (B) FETEM of commercial nanotalc particles, (C) EDS spectrum of indigenous nanotalc particles, and (D) EDS spectrum of commercial nanotalc particles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1. Physicochemical properties of two types of nanotalc particles

	Indigenous Nanotalc (IN)	Commercial Nanotalc (CN)
Average XRD size (nm)	93	89
Average TEM size (nm)	94	91
Surface area (m^2/g)	15.4	15.7
Hydrodynamic size (nm)		
Distilled water	782	735
Cell culture medium	671	643
Zeta potential (mV)	20.3	20.8
Iron content (%)[a]	0.19	0.08

[a]This information is obtained from our previous publication (Akhtar et al., 2010a).

The average TEM size of IN and CN particles were 94 and 91 nm, respectively, which were consistent with the value observed by XRD. The EDS spectra of IN and CN particles are given in Figure 2(C,D), respectively. The presence of Cu and C signals was from the carbon-coated-copper TEM-grid. Presence of iron peaks in both IN and CN particles are in agreement with our previous reports where atomic absorption spectroscopy data showed that 0.19% and 0.08% of iron present in IN and CN particles, respectively (Akhtar et al., 2010 a). The specific surface area of IN and CN particles determined by BET was 15.4 and 15.7 m^2/g respectively.

The physicochemical properties of IN and CN particles are listed in Table 1. All the data from XRD, electron microscopy, and associated techniques was obtained under high vacuum and constitutes the size, morphology, and composition analysis characteristics of nanotalc particles. However, once the nanotalc particles were introduced aqueous media, the sizes changed to approximately 5 to 10 times of the primary size. The average hydrodynamic size of IN and CN particles in distilled water was 782 nm and 735 nm while in cell culture media was 671 and 643 nm, respectively. The higher size of IN and CN particles in aqueous state as compared to XRD and TEM results was due to the tendency of particles to aggregate in the aqueous state. This finding is supported by other investigators (Murdock et al., 2008) and has been briefly discussed in our previous publications (Ahamed et al., 2010a,b). The tendency of particles to form aggregates depends strongly on the surface charge. The particle charge, determined as zeta-potential by laser doppler velocimetry (LDV) was 20.3mV and 20.8 for IN and CN, respectively.

IN and CN Particles Induced Cytotoxicity

We examined the cell viability (MTT assay) and membrane damage (LDH leakage) as cytotoxicity end points. MTT results demonstrated that both IN and CN particles induced significant reduction in cell viability. The MTT reduction

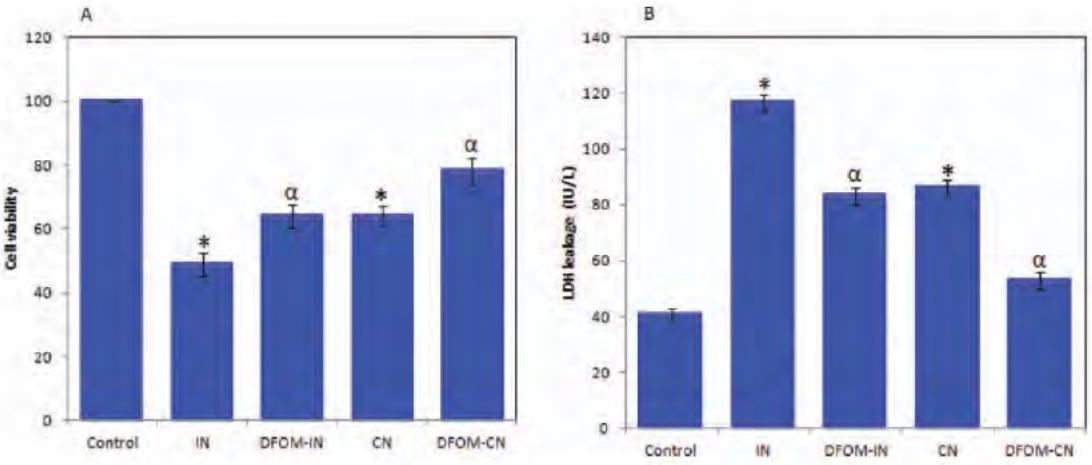

Fig. 3. Comparative effects of nanotalc particles and iron-chelated nanotalc particles on cell viability and LDH release in human lung epithelial A549 cells. Cells were treated with two types of nanotalc particles at the concentration of 200 μg/mL for 48 h. Iron chelator deferoxamine mesylate (DFOM) was co-exposed with nanotalc particles. At the end of treatment MTT and LDH assays were determined as described in materials and methods. (A) MTT assay and (B) LDH assay. Data represented are mean ± SD of three identical experiments made in three replicates. *Statistically significant difference in cell viability reduction and LDH release as compared with the controls ($p < 0.05$ for each). $^{\alpha}$Iron chelation by DFOM significantly reduces the cytotoxicity caused by nanotalc particles ($p < 0.05$ for each). IN; indigenous nanotalc particles, CN; commercial nanotalc particles, DFOM-IN; indigenous nanotalc particles treated with DFOM, DFOM-CN; commercial nanotalc particles treated with DFOM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

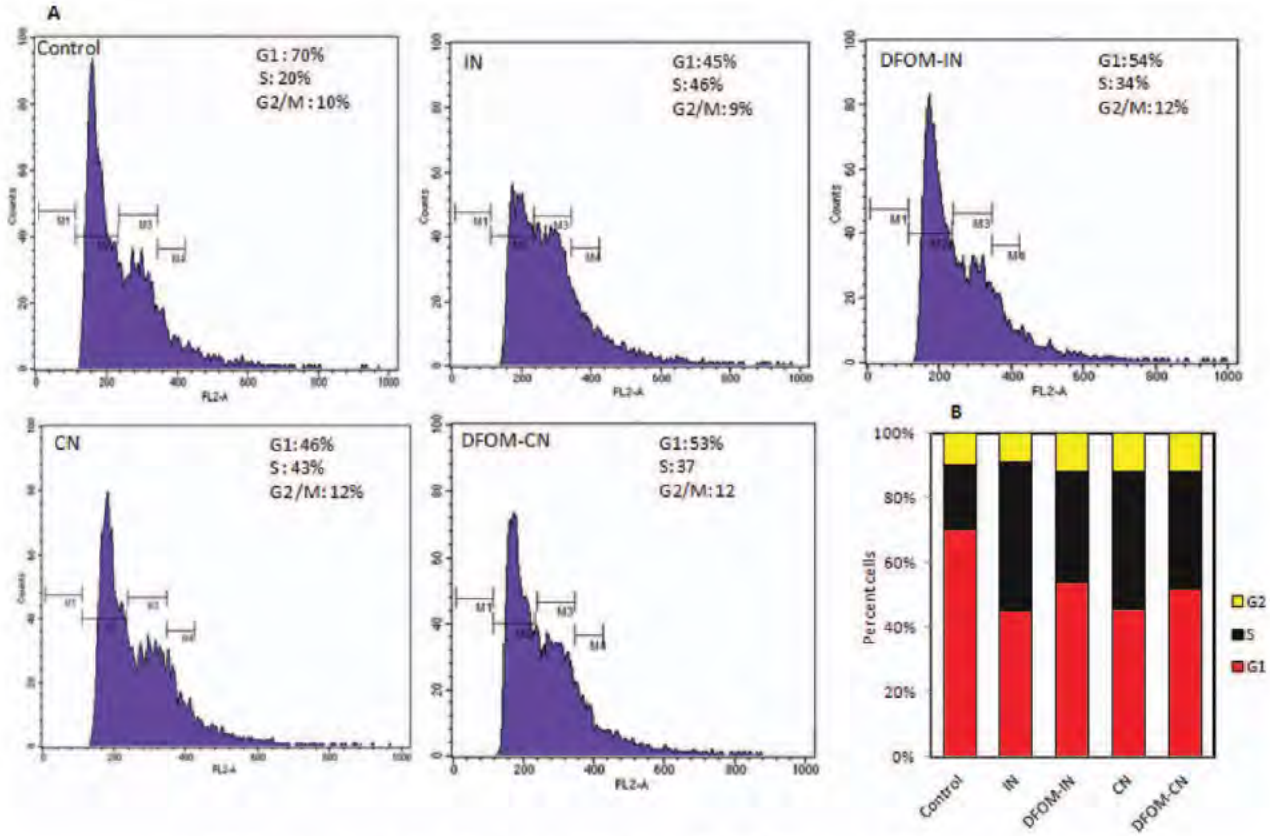

Fig. 4. Comparative effects of nanotalc particles and iron-chelated nanotalc particles on cell cycle in human lung epithelial A549 cells. Cells were treated with two types of nanotalc particles at the concentration of 200 μg/mL for 48 h. Iron chelator deferoxamine mesylate (DFOM) was coexposed with nanotalc particles. At the end of treatment cell cycle was analyzed as described in materials and methods. (A) Raw data generated by flow cytometric analysis of selected representative samples. The *y*-axis denotes cell count and the *x*-axis represents DNA content. M1, M2, M3, and M4 represent the SubG1, G1, S, and G2/M phase, respectively. (B) Percent of the distribution of cells in the G1, S, and G2/M phase of cell cycle. IN; indigenous nanotalc particles, CN; commercial nanotalc particles, DFOM-IN; indigenous nanotalc particles treated with DFOM, DFOM-CN; commercial nanotalc particles treated with DFOM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

observed after 48 h at the concentration of 200 μg/mL was 49% and 64% for IN and CN particles, respectively [Fig. 3(A)]. Both IN and CN particles were also found to induce LDH leakage in A549 cells [Fig. 3(B)]. To determine whether our observed cytotoxicity was due iron content, we treated both IN and CN particles with an iron chelator DFOM and tested the cytotoxic effect of chelated nanotalc particles in A549 cells. Results showed that iron chelated IN and CN particles induce less cytotoxicity than those of non-chelated one (Fig. 3).

IN and CN Particles Induced Cell Cycle Changes

Alteration in the cell cycle phases by IN and CN particles in A549 cells are shown in Figure 4. Both IN and CN particles induced significant S phase arrest. The S phase was 20% in the control. It was changed to 46% and 43% in the cells treated with IN and CN particles respectively. However, iron chelated IN and CN particles exert less effect on cell cycle arrest than those of nonchelated IN and CN particles.

IN and CN Particles Induced Oxidative Stress

ROS generation leads to oxidative damage, which has been reported to be one of the important mechanisms of nanoparticles toxicity (Ahamed et al., 2010c; Ahamed et al., 2011a,b). The potential of IN and CN particles to induce oxidative stress was examined by measuring the ROS, LPO, GSH, SOD, and CAT in A549 cells. Results showed that both IN and CN particles induced the

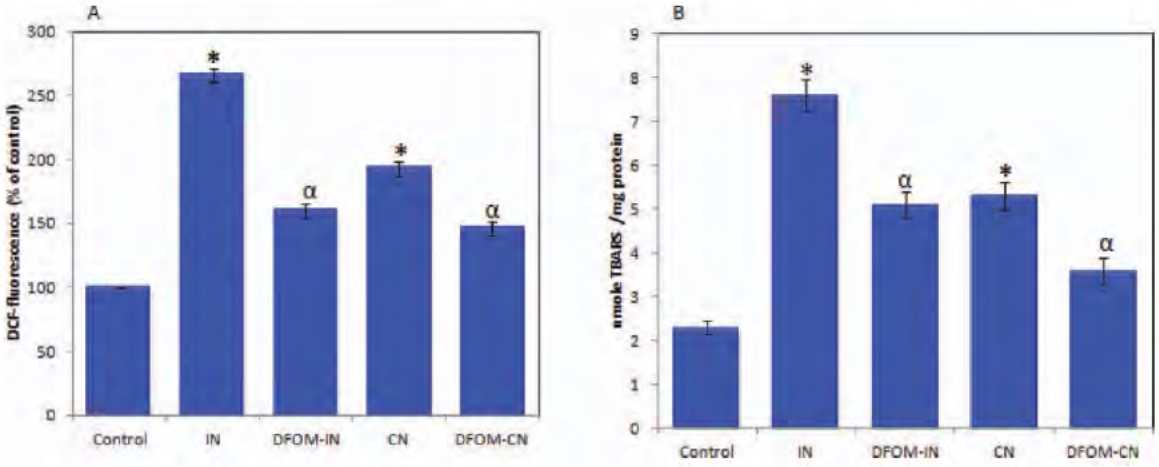

Fig. 5. Comparative effects of nanotalc particles and iron-chelated nanotalc particles on oxidant generations in human lung epithelial A549 cells. Cells were treated with two types of nanotalc particles at the concentration of 200 μg/mL for 48 h. Iron chelator deferoxamine mesylate (DFOM) was coexposed with nanotalc particles. At the end of treatment ROS and LPO levels were determined as described in materials and methods. (A) ROS and (B) LPO. Data represented are mean ± SD of three identical experiments made in three replicates. *Statistically significant difference in ROS and LPO induction as compared with the controls ($p < 0.05$ for each). $^{\alpha}$Iron chelation by DFOM significantly reduces the ROS and LPO induction caused by nanotalc particles ($p < 0.05$ for each). IN; indigenous nanotalc particles, CN; commercial nanotalc particles, DFOM-IN; indigenous nanotalc particles treated with DFOM, DFOM-CN; commercial nanotalc particles treated with DFOM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

intracellular ROS and LPO levels [Fig. 5(A,B)]. Nanotalc particles induced oxidative stress was further evidenced by depletion of GSH, SOD, and CAT [Fig. 6(A,B,C)]. Moreover, chelation of iron from IN and CN particles significantly reduced the oxidative stress due to these particles.

IN and CN Particles Induced Apoptosis

Apoptosis is executed by series of cysteine proteases known as caspases (Takadera and Ohyashiki, 2007; Tang et al., 2010). Caspase-9 activation is dependent on the release of cytochrome c from mitochondria to form the apoptosome which in turn activates caspase-3. In the present study, significant higher activity of caspase-3 enzyme was observed suggesting the involvement of caspase cascade in IN and CN particles induced apoptosis in A549 cells [Fig. 7(B)]. Figure 7(B) shows that in untreated cells, the DNA was intact whereas the cells treated with IN and CN particles had started apoptotic DNA fragmentation. Besides, iron chelation from IN and CN particles induced less DNA fragmentation as compared with the nonchelated particles.

Taken together, our data highlight the role of iron contaminant present in IN and CN particles in causing the cytotoxicity, oxidative stress, and apoptosis in human lung epithelial cells.

DISCUSSION

Characterization of physicochemical properties of nanoparticles has been suggested in the nanotoxicology research (Murdock et al., 2008; Li et al., 2011). Several parameters including shape, size, crystal structure, purity, hydrodynamic size, aggregation of particles, and aqueous stability have already been suggested (Nel et al., 2006; Yu et al., 2009). In this study, we employed XRD, TEM, EDS, BET, and DLS techniques to characterize the physicochemical properties of IN and CN particles. XRD and TEM results indicated that both IN and CN particles were crystalline, highly aggregated, and having the iron content as a contaminant. Aggregation and stability of nanoparticles in aqueous state are major concerns in nanotoxicity research. Both IN and CN particles were also aggregated in water and cell culture media as well. Zeta potential data also showed that the aqueous suspension of both IN and CN particles were not much stable in aqueous state. The hydrodynamic size of nanotalc particles was found to be approximately seven to eight times higher than those calculated from TEM and XRD. The higher size of nanoparticles in aqueous suspension as compared with XRD and TEM sizes might be due to the tendency of particles to aggregate in aqueous state. This finding is supported by other investigators (Bai et al., 2009) and has been briefly discussed in our previous publication (Ahamed et al., 2010b).

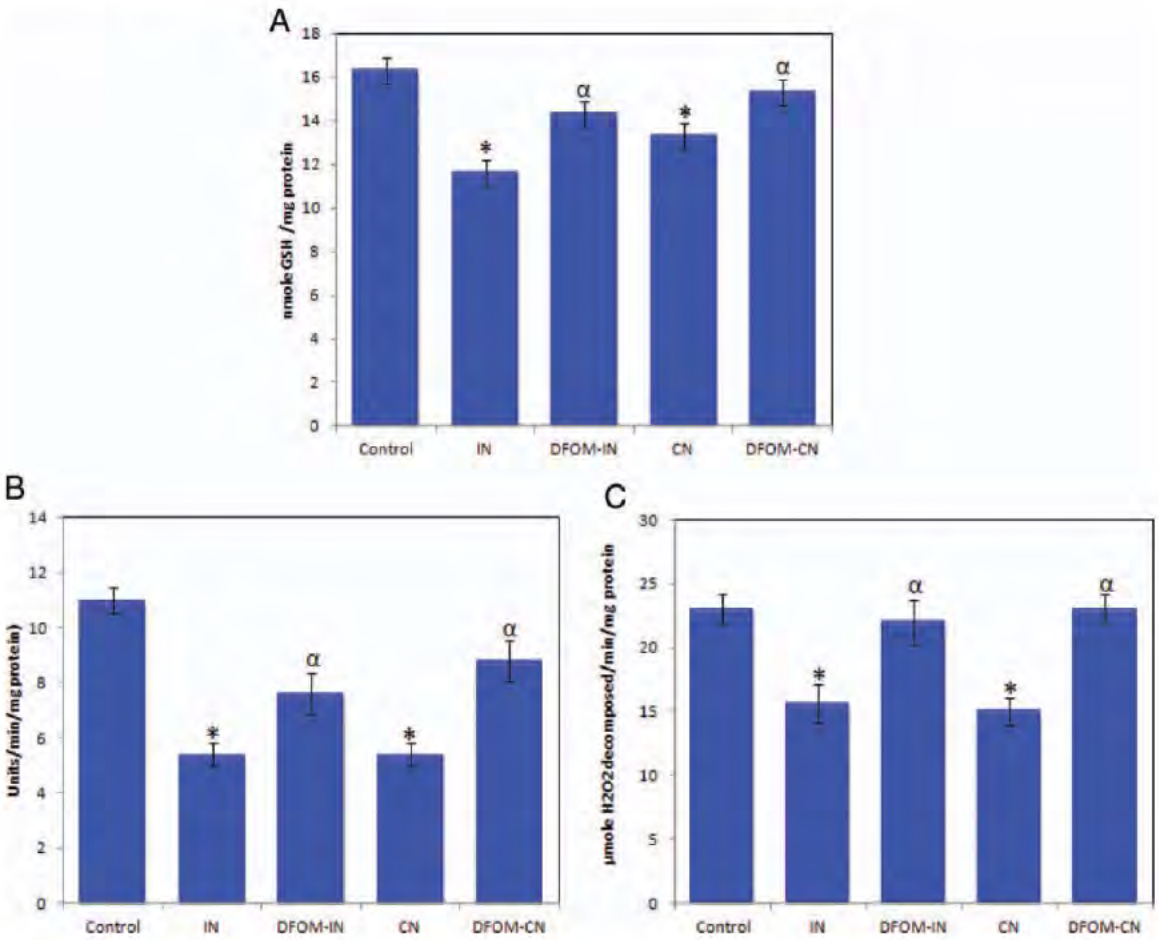

Fig. 6. Comparative effects of nanotalc particles and iron-chelated nanotalc particles on antoxidants reduction in human lung epithelial A549 cells. Cells were treated with two types of nanotalc particles at the concentration of 200 μg/mL for 48 h. Iron chelator deferoxamine mesylate (DFOM) was coexposed with nanotalc particles. At the end of treatment GSH, SOD, and CAT levels were determined as described in materials and methods. (A) GSH, (B) SOD, and (C) CAT. Data represented are mean ± SD of three identical experiments made in three replicates. *Statistically significant difference in GSH, SOD, and CAT reduction as compared to the controls ($p < 0.05$ for each). $^{\alpha}$Iron chelation by DFOM significantly induces the GSH, SOD, and CAT depletion caused by nanotalc particles ($p < 0.05$ for each). IN; indigenous nanotalc particles, CN; commercial nanotalc particles, DFOM-IN; indigenous nanotalc particles treated with DFOM, DFOM-CN; commercial nanotalc particles treated with DFOM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In this study, we observed that IN and CN particles induced cell viability reduction and membrane damage in A549 cells. Both IN and CN particles also induced the cell cycle arrest in the S phase leading to apoptosis. In a previous study, S phase arrest was observed in mouse peritoneal macrophages (RAW264.7) exposed to silver nanoparticles (Park et al., 2010), and S phase arrest was also observed in human lung epithelial cells exposed to carbon black particles coated with benzo(a)pyrene (Mroz et al., 2007). Asharani et al. (2009) reported that starch-coated silver NPs induced G2/M phase arrest and DNA damage in human glioblastoma cells and fibroblasts. A perturbation of the cell cycle preceded by a reduction in cell viability associated with accumulation of cells in S phase leading to cell death is typical of compounds inhibiting DNA synthesis (Binkova et al., 2000; Park et al., 2010).

Cellular integrity is affected by oxidative stress when the production of ROS overwhelms antioxidant defense mechanism (Halliwell and Gutteridge, 1990). Our results showed that both IN and CN particles induce oxidant levels and deplete the antioxidant levels in human lung epithelial (A549) cells. LPO and ROS were significantly higher while the antioxidant GSH was significantly lower in cells treated with IN and CN particles. Antioxidant enzymes SOD and

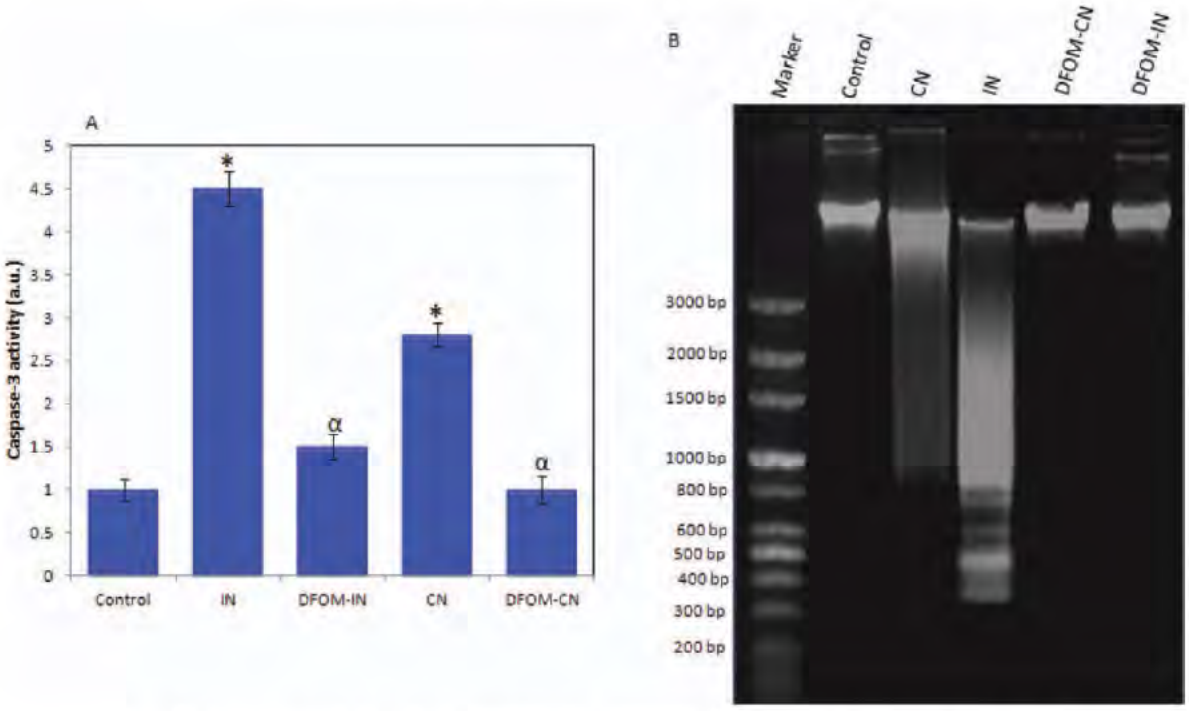

Fig. 7. Comparative effects of nanotalc particles and iron-chelated nanotalc particles on apoptotic markers in human lung epithelial A549 cells. Cells were treated with two types of nanotalc particles at the concentration of 200 μg/mL for 48 h. Iron chelator deferoxamine mesylate (DFOM) was coexposed with nanotalc particles. At the end of treatment DNA ladder and caspase-3 activity were determined as described in materials and methods. (A) Caspase-3 activity. Data represented are mean ± SD of three identical experiments made in three replicates. *Statistically significant difference in caspase-3 activation as compared with the controls ($p < 0.05$ for each). [α]Iron chelation by DFOM significantly reduces the activity of caspase-3 by nanotalc particles ($p < 0.05$ for each). (B) Representative image of DNA fragmentation. IN; indigenous nanotalc particles, CN; commercial nanotalc particles, DFOM-IN; indigenous nanotalc particles treated with DFOM, DFOM-CN; commercial nanotalc particles treated with DFOM. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CAT levels were also significantly lower in exposed cells. GSH constitutes the first line of the cellular defense mechanism against oxidative injury and is the major intracellular redox buffer in ubiquitous cell types (Meister, 1989). GSH acts as a cosubstrate in the GSH peroxidase-catalyzed reduction of hydrogen peroxide or lipid peroxides (Forman et al., 1997) leading to its depletion. Previous studies demonstrated that ROS generation following GSH depletion caused mitochondrial damage (Martensson et al., 1989), which has been implicated in apoptosis (Green and Reed, 1998). Enzymes such as SOD and CAT are meant for nullifying cellular oxidative stress. SOD catalyzes the dismutation of superoxide anion (O_2) to hydrogen peroxide (H_2O_2). CAT reduces hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) (Claiborne, 1985).

The activity of caspase-3 enzyme was significantly higher in cells treated with IN and CN particles. Apoptotic DNA fragmentation was observed in cells exposed to IN and CN particles. Caspases are activated in response to diverse cell death stimuli and ultimately dismantle the cell through restricted proteolysis of numerous cellular proteins that (Timmer and Salvesen, 2007). The activated caspase-3 is capable of autocatalysis as well as cleaving and activating other members of the caspase family, leading to rapid and irreversible apoptosis (Wang et al., 1996). Our previous studies also reported that different types of nanoparticles have potential to induce apoptosis in different kind of cells (Ahamed et al., 2010a; 2010b; 2010c; Ahamed et al., 2010b,c; 2011a).

In the toxicity mechanism of minerals, the iron content has been a key factor. In the present study, EDS analysis showed the presence of iron contamination in both IN and CN particles. These results are in agreement with our previous report where atomic absorption spectroscopy showed the presence of 0.19% and 0.08% of iron in IN and CN particles respectively (Akhtar et al., 2010a). Iron-dependent

ROS generation from fibers results in the generation of hydroxyl radicals through the Fenton reaction and the Haber-Weiss cycle. Iron-dependent ROS generation requires redox cycling of iron and does not necessarily require H_2O_2 or ROS (Halliwell and Gutteridge, 1990). The differential amount of iron present in the two types of nanotalc particles prompted us to investigate the role of iron by sequestering them with an iron chelator, deferoxamine mesylate (DFOM). Sequestering of redox active iron from IN and CN particles by DFOM caused significantly less cytotoxicity, oxidative stress, and genotoxicity than those of the nonchelated IN and CN particles. Similarly, incubation of crocidolite or chrysotile fibers overnight with deferoxamine (5 mM) to inactivate iron catalyzed oxygen radical production also significantly decreased asbestos-induced apoptosis (Broaddus et al., 1996). The role of iron in minerals such as asbestos or silica has been well reported in inflammation and carcinogenesis (Ghio et al., 1992; Hardy and Aust. 1995). Zastawny et al. (1995) have reported on DNA base modifications and membrane damage in cultured mammalian cells treated with iron itself. Similarly, intracellular iron was found to play a critical role in hydrogen peroxide-induced DNA damage (Barbouti et al., 2001). It is also worth to mention that IN particles caused higher toxicity to A549 cells than those of CN particles. This might be due to higher amount of iron present in IN particles (0.19%) as compared with the CN particles (0.08%).

In conclusion, both IN and CN particles significantly induced cytotoxicity, oxidative stress, and apoptosis in human lung epithelial cells. Further, chelation of iron from IN and CN particles by deferoxamine mesylate treatment caused significantly less toxicity as compared to non-chelated IN and CN particles. Therefore, iron content plays a significant role in the toxicity of IN and CN particles, which may be mediated through ROS generation and oxidative stress. This study suggests that one must be very careful regarding the metal impurities like iron present in nanotalc particles before commercial and industrial applications.

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Exhibit 93

Alterations in Gene Expression in Human Mesothelial Cells Correlate with Mineral Pathogenicity

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Human mesothelial cells (LP9/TERT 1) were exposed to low and high (15 and 75 $\mu m^2/cm^2$ dish) equal surface area concentrations of crocidolite asbestos, nonfibrous talc, fine titanium dioxide (TiO_2), or glass beads for 8 or 24 hours. RNA was then isolated for Affymetrix microarrays, GeneSifter analysis and QRT PCR. Gene changes by asbestos were concentration and time dependent. At low nontoxic concentrations, asbestos caused significant changes in mRNA ex pression of 29 genes at 8 hours and of 205 genes at 24 hours, whereas changes in mRNA levels of 236 genes occurred in cells exposed to high concentrations of asbestos for 8 hours. Human primary pleural mesothelial cells also showed the same patterns of increased gene expression by asbestos. Nonfibrous talc at low concentrations in LP9/TERT 1 mesothelial cells caused increased expression of 1 gene Activating Transcription Factor 3 (*ATF3*) at 8 hours and no changes at 24 hours, whereas expression levels of 30 genes were elevated at 8 hours at high talc concentrations. Fine TiO_2 or glass beads caused no changes in gene expression. In human ovarian epithelial (IOSE) cells, asbestos at high concentrations elevated expression of two genes (*NR4A2, MIP2*) at 8 hours and 16 genes at 24 hours that were distinct from those elevated in mesothelial cells. Since *ATF3* was the most highly expressed gene by asbestos, its functional importance in cytokine production by LP9/TERT 1 cells was assessed using siRNA approaches. Results reveal that *ATF3* modulates production of in flammatory cytokines (IL 1β, IL 13, G CSF) and growth factors (VEGF and PDGF BB) in human mesothelial cells.

Keywords: mesothelioma; crocidolite asbestos; talc; titanium dioxide; gene profiling

A myriad of natural and synthetic fibers and particles, including nanomaterials, are being introduced into the workplace and environment, and *in vitro* screening tests on human cell types are needed to predict their toxicity and mechanisms of action, especially in target cells of disease. Asbestos is a group of well characterized fibrous minerals that are associated with the development of nonmalignant (asbestosis) and malignant (lung cancers, pleural, and peritoneal mesotheliomas) diseases in occu pational cohorts (1 3), yet the molecular mechanisms of asbestos related diseases are poorly understood. Although it is widely acknowledged that fibrous geometry, surface and chemical com position, and durability are important features in the development of asbestos associated diseases, how these contribute to cell toxicity and transformation are unclear. Moreover, the early molecular events leading to injury by asbestos fibers and other pathogenic or innocuous particulates in human cells that may be targets for the development of disease remain enigmatic.

CLINICAL RELEVANCE

Results of work here suggest that transcriptional profiling can be used to reveal molecular events by mineral dusts that are predictive of their pathogenicity in mesothelioma.

The objective of work here was to compare acute toxicity and gene expression profiles of crocidolite asbestos, the type of asbestos most pathogenic in the causation of human mesothe lioma (3, 4), to nonfibrous talc, fine titanium dioxide (TiO_2), and glass beads in a contact inhibited, hTERT immortalized human mesothelial cell line (5). In comparative studies, we also evalu ated toxicity of particulates and gene expression changes in a contact inhibited SV40 Tag immortalized human ovarian epi thelial cell line (IOSE) (6). This cell type is not implicated in asbestos induced diseases, but is occasionally linked to inflam mation and the development of ovarian cancer after use of tal cum powder in the pelvic region, although such links are highly controversial (7).

Although most studies have evaluated the biological effects of particles and fibers on an equal mass or weight basis, the number, surface area, and reactivity of particulates at equal weight concentrations may be vastly different. Moreover, recent *in vitro* (8, 9) and *in vivo* (10 12), studies have confirmed that toxicity, oxidative stress, and inflammatory effects of ultrafine and other particles are related directly to surface area. For these reasons, and to avoid possible confounding alterations in gene expression or toxicity that might reflect or be masked in cells in different phases of the cell cycle, we introduced particulates at equal surface areas to confluent monolayers of human meso thelial (LP9/TERT 1) and human ovarian epithelial (IOSE) cells in a maintenance medium. Moreover, our studies included a nonfibrous talc sample and fine TiO_2 and glass particles, both traditionally used as nontoxic and nonpathogenic control par ticles in *in vitro* and animal experiments (reviewed in Refs. 13 and 14). Our studies provide novel insight into the early molecular events and responses occurring in human cells after exposure to asbestos and these materials.

MATERIALS AND METHODS

Human Mesothelial and Ovarian Epithelial Cell Cultures

Human mesothelial LP9/TERT 1 (LP9) cells, an hTERT immortal ized cell line phenotypically and functionally resembling normal human mesothelial cells (5), were obtained from Dr. James Rhein wald (Dana Farber Cancer Research Institute, Boston, MA). Human pleural mesothelial cells (NYU474) were isolated surgically from

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This article contains microarray data which can be found as a repository using the accession number GSE14034.

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cancer free patients by Dr. Harvey Pass (New York University, New York, NY). Briefly, tissue sample 2 × 2 cm^2 was harvested into saline solution and rinsed immediately with PBS (1×) and Dulbecco's modified Eagle's medium (DMEM) (1×). The tissue was then digested with 0.2% Collagenase type 1 (MP Biomedical Inc., Solon, OH) for 3 hours at 37°C. Finally, the digested tissue was scraped and cells collected were centrifuged for 5 minutes at 300 × *g*. The cell pellet thus obtained was resuspended in DMEM containing 10% fetal bovine serum (FBS) and 2% penicillin streptomycin, transferred into 6 well plate, and allowed to grow at 5% CO_2 and 37°C. Mesothelial cells were characterized by staining with calretinin antibody. An SV40 Tag immortalized, anchorage dependent human ovarian epithelial cell line (IOSE 398) (6) was a kind gift from Dr. Nelly Auersperg (Canadian Ovarian Tissue Bank, University of British Columbia, Vancouver, BC, Canada). LP9/TERT 1 cells were maintained in 50:50 DMEM/F 12 medium containing 10% FBS, and supplemented with pen icillin (50 units/ml), streptomycin (100 μg/ml), hydrocortisone (100 μg/ml), insulin (2.5 μg/ml), transferrin (2.5 μg/ml), and selenium (2.5 μg/ml). IOSE cells were maintained in 50:50 199/MCB105 medium containing 10% FBS and 50 μg/ml gentamicin. Cells at near confluence were switched to maintenance medium containing 0.5% FBS for 24 hours before par ticulate exposure. NYU474 cells were grown to near confluence in DMEM containing 10% FBS and supplemented with penicillin (50 units/ml) and streptomycin (100 μg/ml).

Characterization of Mineral Preparations

The physical and chemical characterization of the NIEHS reference sample of crocidolite asbestos has been reported previously (15). The surface area of asbestos fibers and particles was measured using nitrogen gas sorption analysis to allow computation of identical amounts of sur face areas of particulates to be added to cells. Fiber and particle size dimensions were determined by scanning electron microscopy (SEM) as described previously (16). In addition, talc was examined using field emis sion scanning electron microscopy (FESEM) and transmission electron microscopy (TEM). The chemical composition, surface area, mean size, and source of each particulate preparation is presented in Table 1.

Introduction of Particulates to Cells

After sterilization under ultraviolet light overnight to avoid endotoxin and microbial contamination, particulates were suspended in HBSS at 1 mg/ml, sonicated for 15 minutes in a water bath sonicator, and triturated five times through a 22 gauge needle. This suspension was added to cells in medium.

SEM to Determine Particulate/Cell Interactions

Cells were grown on Thermonox plastic cover slips (Nalge Nunc International, Naperville, IL), exposed to particulates for 24 hours, and then processed for SEM as described previously (16). After samples were critical point dried, they were mounted on aluminum specimen stubs and dried before being sputter coated with gold and palladium in a Polaron sputter coater (Model 5100; Quorum Technol ogies, Guelph, ON, Canada) and examined on a JSM 6060 scanning electron microscope (JEOL USA, Inc., Peabody, MA).

Cell Viability Studies

After 24 hours, cells were collected with Accutase cell detachment reagent, and final cell suspensions in Accutase/complete medium/HBSS were mixed with 0.4% trypan blue stain, which is retained by dead cells. After 5 minutes, unstained cells were counted using a hemocytometer to determine the total number of viable cells per dish.

Based on the results of cell viability studies, asbestos and nonfibrous talc were evaluated in LP9 mesothelial cells for changes in gene expression at both low and high concentrations (15 and 75 $\mu m^2/cm^2$ dish) at 8 hours, and at low concentrations of minerals (15 $\mu m^2/cm^2$ dish) at 24 hours. These concentrations did not cause morphologic or toxic cellular changes at these time points. Negative control groups included cells exposed to fine TiO_2 (15 $\mu m^2/cm^2$ dish) at 8 and 24 hours and glass beads (75 $\mu m^2/cm^2$) at 24 hours. In IOSE cells, gene expression of all particulates was evaluated at 75 $\mu m^2/cm^2$ at 8 and 24 hours, as preliminary experiments revealed that no significant changes in mRNA levels were observed at 15 $\mu m^2/cm^2$ dish of asbestos. In NYU474 human mesothelial cells, QRT PCR was used to validate a selected subset of gene expression changes identified by arrays in LP9/TERT 1 cells. Cells were exposed to 15 and 75 $\mu m^2/cm^2$ asbestos for 24 hours, and 8 genes highly expressed in LP9 cells were examined by QRT PCR (*see below*).

RNA Preparation

Total RNA was prepared using an RNeasy Plus Mini Kit according to the manufacturers' protocol (Qiagen, Valencia, CA), as previously described (17).

Affymetrix Gene Profiling

Microarrays were performed on samples from three independent experiments. All cell types, time points, and mineral types and con cenrations were included in all three experiments. For each experi ment, $n = 3$ dishes were pooled into one sample per treatment group. Each of the pooled samples was analyzed on a separate array (i.e., $n = 3$ arrays per condition [3 independent biological replicates]). All proce dures were performed by the Vermont Cancer Center DNA facility using standard Affymetrix protocol as previously described (14, 17). Each probe array, Human U133A 2.0 (Affymetrix, Santa Clara, CA) was scanned twice (Hewlett Packard GeneArray Scanner, Palo Alto, CA), the images overlaid, and the average intensities of each probe cell compiled. Microarray data were analyzed using GeneSifter software (VizX Labs, Seattle, WA). This program used a "*t* test" for pairwise comparison and a Benjamini Hochberg test for false discovery rate (FDR 5%) to adjust for multiple comparisons. A 2 fold cutoff limit was used for analysis.

Quantitative Real Time PCR

Total RNA (1 μg) was reverse transcribed with random primers using the Promega AMV Reverse Transcriptase kit (Promega, Madison, WI) according to the recommendations of the manufacturer, as described previously (17). In NYU474 mesothelial cells, eight genes (*ATF3*, *SOD2*, *PTGS2*, *FOSB*, *TFPI2*, *PDK4*, *NR4A2*, and *IL 8*) most highly expressed in LP9 cells were evaluated using the ΔΔCt method. Duplicate or triplicate assays were performed with RNA samples isolated from at least three independent experiments. The values obtained from cDNAs and hypoxanthine phosphoribosyl transferase (*hprt*) controls provided relative gene expression levels for the gene locus investigated. The primers and probes used to validate gene expression as observed in microarrays were purchased from Applied Biosystems (Foster City, CA).

TABLE 1. CHARACTERIZATION OF PARTICULATES

Name	Chemical Composition	Mean Surface Area ± SE (m^2/g)	Mean Size (μm)*	Source
Crocidolite Asbestos	$Na_2Fe_3^{2+}Fe_2^{3+}Si_8O_{22}(OH)_2$	14.97 ± 0.605	7.4 × 0.25	NIEHS Reference Sample
Talc (MP 10-52)†	$Mg_3Si_4O_{10}(OH)_2$	16.03 ± 0.654	1.1	Barrett's Minerals, Inc.
Titanium Dioxide	TiO_2	9.02 ± 0.185	0.69	Fisher Scientific
Glass Beads	SiO_2	2.78 ± 0.215	2.06	Polysciences Inc.

* Length X width for crocidolite asbestos, and diameter for nonfibrous talc, TiO_2, and glass beads.

† Although standard reference samples of asbestos and some particulates are available for use by the scientific community, reference samples of talc currently do not exist. For these reasons, the nonfibrous talc sample was also characterized for physical properties, particle size distribution (0.70 μm minimum to 1.20 μm maximum), and chemical/mineralogical (talc 95%, chlorite 4.5–5%, dolomite 0.3%) composition. For complete analysis or obtaining samples, please contact Brooke Mossman, Mark Ellis (markellis@ima-na.org), or Michelle Wyart at EUROTALC (mwyart@ima-europe.eu).

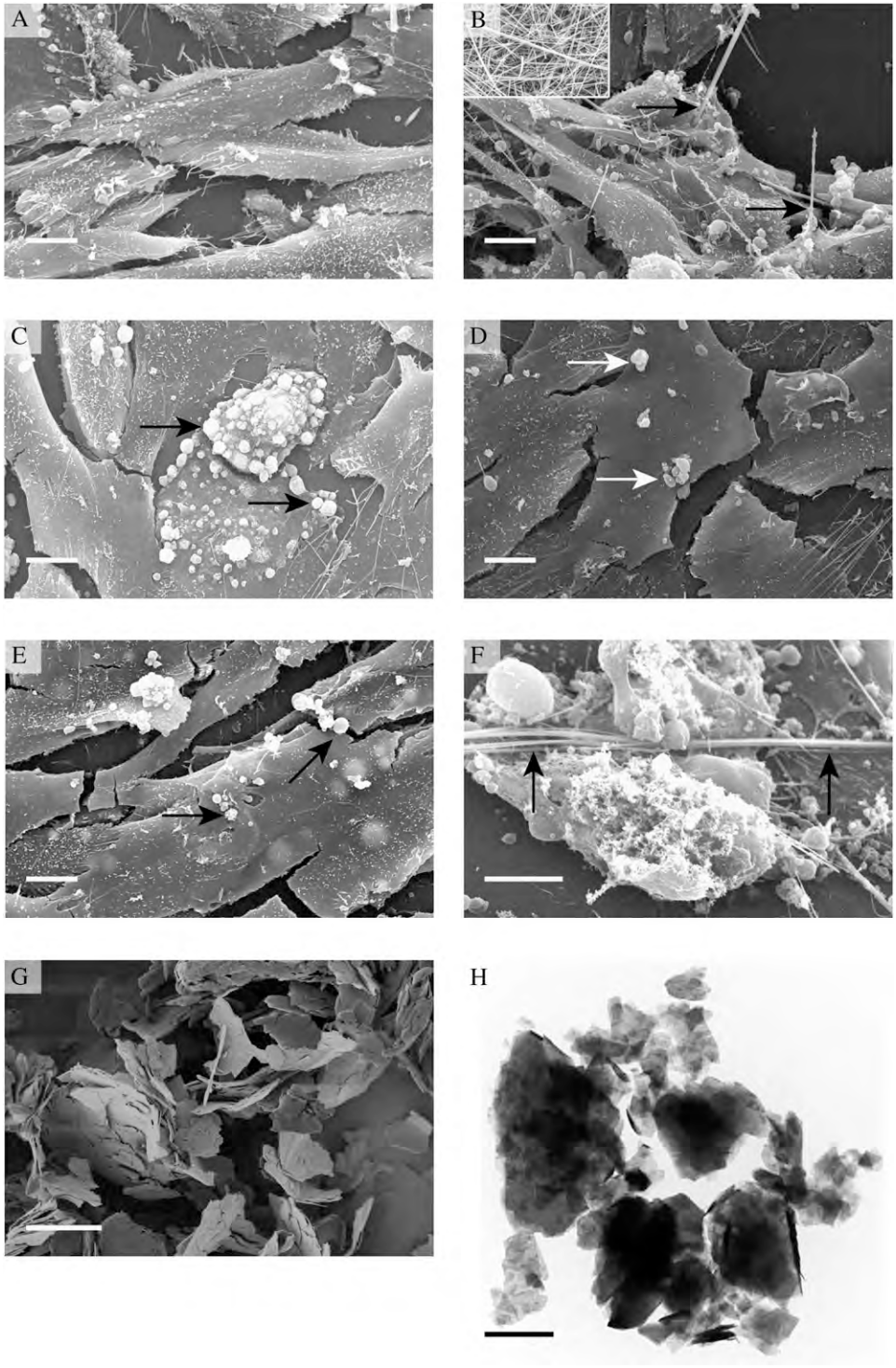

Figure 1. Interaction of fibers and particles with (*A E*) LP9/TERT 1 human mesothelial cells and (*F*) IOSE ovarian epithelial cells after 24 hours of exposure to (*B, E, F*) high and (*C, D*) low concentrations of particulates. (*G*) Field emission scanning electron microscopy (FESEM) and (*H*) transmission electron microscopy (TEM) show structure of nonfibrous talc. (*A*) Morphology of unexposed near confluent LP9/TERT 1 cells. (*B*) Membrane blebbing and piling up of cells in response to crocidolite asbestos (*arrows*). (C) Nonfibrous talc and (*D*) fine TiO_2 (*arrows*) on cell surface. (*E*) Single and small clumps of glass beads on plasma membrane. (*F*) Interaction of asbestos fibers (*arrows*) with IOSE cells that exhibit an exudate and membrane ruffling in response to fibers. *Bars* 10 μm. (*G*) FESEM and (*H*) TEM showing morphology of platy talc bulk material. *Bars* 2 μm.

Transfection of LP9 Cells with siRNA

On Target plus Non targeting siRNA #1 (scrambled control), and On Target plus SMART pool human *ATF3* siRNA (100 nM; Dharmacon, Lafayette, CO) were transfected into LP9 cells at near confluence using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. The efficiency of *ATF3* knockdown was determined by QRT PCR after 48 and 72 hours.

Bio Plex Analysis of Cytokine and Chemokine Concentrations in Medium of LP9/TERT 1 Cells

To quantify cytokine and chemokine levels in conditioned medium of cells transfected with siATF3 or scrambled control and exposed to asbestos for 24 hours, a multiplex suspension protein array was performed using the Bio Plex protein array system as described previously (17) and a Human Cytokine 27 plex panel (Bio Rad, Hercules, CA). Three biological replicates were used for each treatment group.

Statistical Analysis

Data from QRT PCR and cell viability assays were evaluated by ANOVA using the Student Neuman Keul's procedure for adjustment of multiple pairwise comparisons between treatment groups or using the nonparametric Kruskal Wallis and Mann Whitney tests. Differences with P values ≤ 0.05 were considered statistically significant.

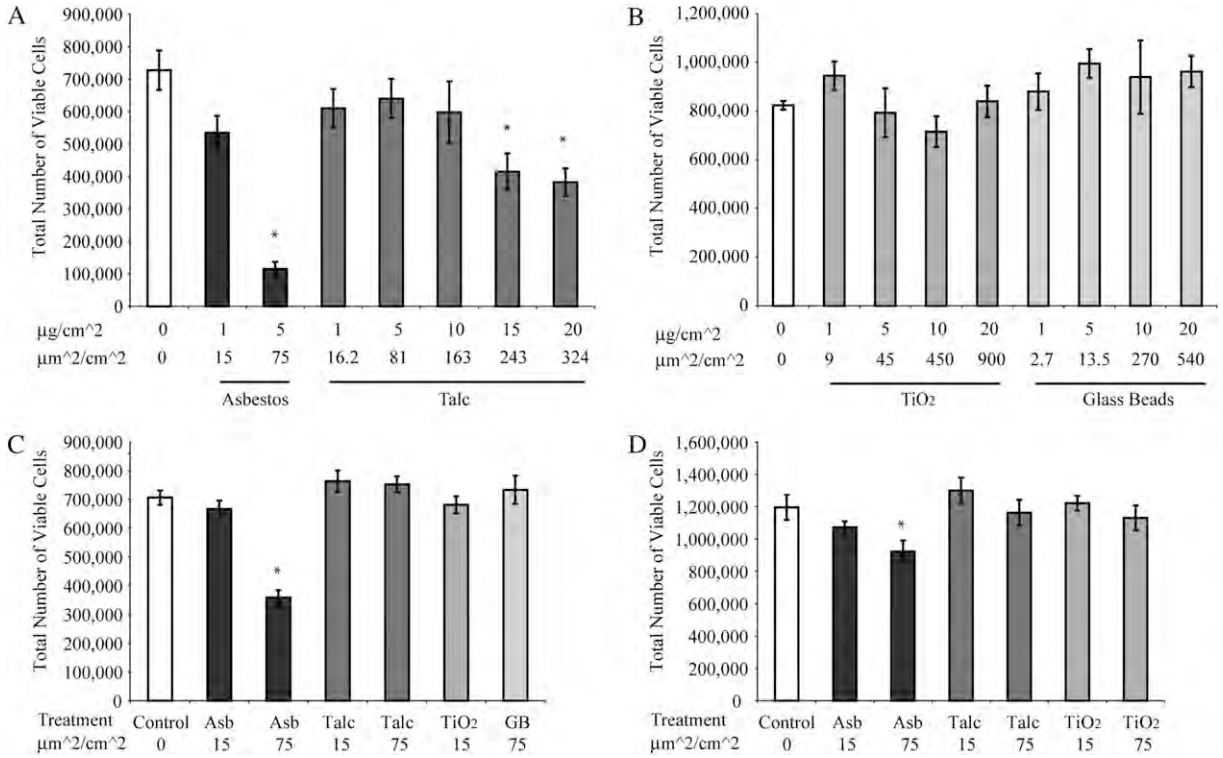

Figure 2. Cell viability after 24 hours of exposure to asbestos fibers and particles in (*A C*) LP 9/TERT 1 and (*D*) IOSE (D). Mean +/− SE of 1 (A, B) or 3 (C, D) individual experiments where *n* 3 per group per experiment. * $P \leq 0.05$ compared with untreated (0) groups.

RESULTS

Characterization of Particulate Preparations

Table 1 shows the major chemical formulas of crocidolite asbestos fibers (defined as having a greater than 3:1 length to width ratio) and particle samples used in experiments, although trace amounts of other elements occur in the NIEHS asbestos standards (15). In addition, we examined the morphology and cellular interactions of asbestos fibers, talc, and other particles using SEM (Figure 1). These studies revealed that only high (75 $\mu m^2/cm^2$) surface area concentrations of asbestos caused membrane blebbing and other toxic manifestations in cells (Figures 1B and 1F). In contrast, particles of nonfibrous talc (Figure 1C), fine TiO_2 (Figure 1D), and glass beads (Figure 1E) were nontoxic. Both asbestos fibers and particles were observed on the cell surface and were encompassed by cells. Nonfibrous talc occurred in platy particles that were uniform in appearance as viewed by FESEM (Figure 1G) and TEM (Figure 1H).

Asbestos Fibers at High Concentrations Are Toxic to LP9/TERT 1 Human Mesothelial Cells and Less So to Ovarian Epithelial Cells in Contrast to Particle Preparations

Figure 2 shows the results of trypan blue exclusion tests in LP9/TERT 1 and IOSE cells. In LP9/TERT 1 cells (Figures 2A 2C), asbestos at high surface area concentrations (75 $\mu m^2/cm^2$) caused significant decreases (50 80%) in cell viability that were more striking than those observed in IOSE cells (Figure 2D). Nonfibrous talc at 75 $\mu m^2/cm^2$ was nontoxic, and significant increases in toxicity were only achieved with addition of talc at $\geq$ 3 fold higher concentrations in LP9/TERT 1 cells (Figure 2A), but not in IOSE cells (data not shown). Neither TiO_2 nor glass beads were significantly toxic to either cell type over a range of concentrations (Figure 2B).

Asbestos Fibers, but Not Particle Preparations, Cause Dose and Time Related Changes in Gene Expression in Human LP9 Mesothelial Cells

Figure 3 shows a summary of significantly increased or de creased (> 2 fold compared with untreated controls) gene expression by asbestos (Figures 3A 3C) and nonfibrous talc (Figure 3D) in LP9/TERT 1 cells as well as the classification of genes by ontology. These studies revealed that gene expression changes by low concentrations of asbestos were less (29 increases) than at high concentrations (236 alterations including decreases) at 8 hours. Moreover, numbers of significant mRNA level alterations (205) at low concentrations of asbestos in creased over time. In contrast, fewer numbers (30) of gene expression increases were observed at high concentrations of talc at 8 hours compared with identical surface areas of asbestos (236 changes), and no decreases in gene expression were observed. No significant alterations in gene expression were observed with low concentrations of talc at 24 hours or with TiO_2 or glass beads at either concentration or time point (data not shown). The major genes affected by asbestos or talc in LP9/TERT 1 cells are listed in Tables 2 4. This information reveals that the fold increases in common genes expressed by asbestos treated cells increase in a dose related fashion at 8 hours. Although dose responses were observed with talc at 8 hours, the numbers of significant gene increases as well as fold in creases were less than that observed with asbestos and decreased over time. Since mRNA expression of *ATF3* and *IL8* were increased by either asbestos or talc in LP9/TERT 1 cells, the increased expression of these genes was verified by QRT PCR in mineral exposed cells as compared with untreated control cells (Figure 4).

In NYU474 cells, QRT PCR was used to validate that eight asbestos induced genes in LP9 cells were up regulated in

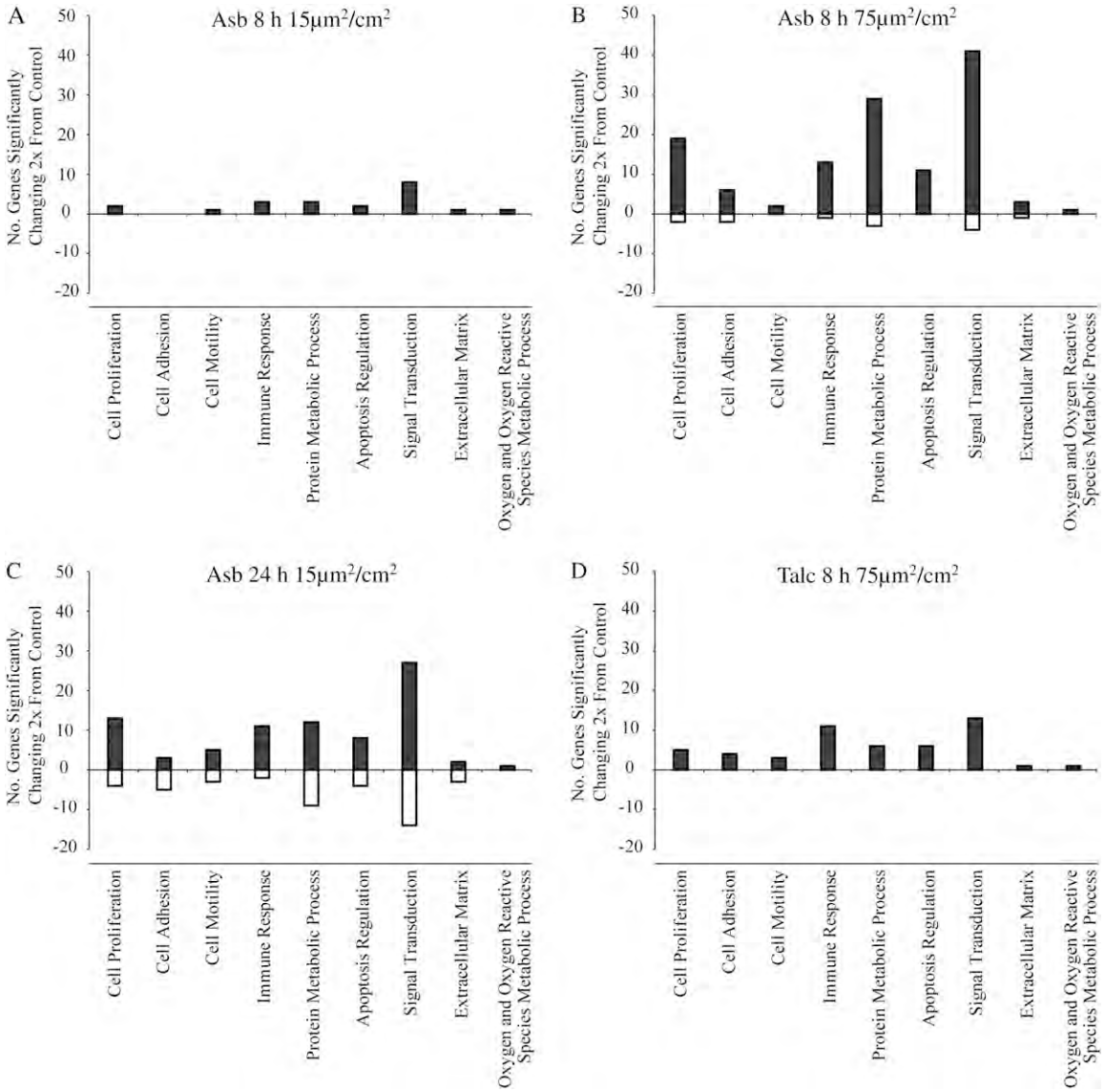

Figure 3. Numbers of changes ($P \leq 0.05$) in gene expression and classification by ontology in LP9/TERT 1 cells after exposure to (*A C*) crocidolite asbestos or (*D*) nonfibrous talc.

normal human mesothelial cells (*ATF3*, *PTGS2* or *COX2*, *FOSB*, *IL8*, *NR4A2*, and *TFPI2*). Results showed that mRNA levels of six of the eight genes evaluated were increased in a dose responsive fashion after exposure to asbestos for 24 hours (Figure 5).

IOSE Ovarian Epithelial Cells Exhibit Few Gene Expression Changes in Response to Asbestos

In contrast to LP9/TERT 1 and NYU474 mesothelial cells, IOSE cells showed no significant gene up regulation or down regulation in response to lower concentrations of asbestos at 8 or 24 hours (data not shown). At high concentrations of asbestos at 8 hours, mRNA levels of only two genes (*NR4A2* and *CXCL2* or *MIP2*) were increased in comparison to untreated IOSE cells (Table 4). At 24 hours, high concentrations of asbestos caused less than 4 fold increases in expression of only 16 genes, and decreased expression of 1 gene, *Profilin 1* (data not shown). No significant mRNA changes were observed with nonfibrous talc, fine TiO_2 or glass beads at either time point.

Inhibition of *ATF3* by siRNA Alters Asbestos Induced Cytokines in LP9/TERT 1 Cells

Since *ATF3* was a common gene up regulated by asbestos in mesothelial cells its functional role in cytokine production in LP9 cells was evaluated. As shown in Figure 6A, *ATF3* was success fully inhibited in LP9/TERT 1 cells using siATF3 as described in MATERIALS AND METHODS. Cells transfected with control siRNA or siATF3 were then exposed to asbestos (75 $\mu m^2/cm^2$ $n = 3$) for 24 hours, and medium was collected and analyzed for cytokines and growth factors using Bio Plex analyses. Inhibition of *ATF3* altered levels of asbestos induced inflammatory cytokines (IL 1β, IL 13, G CSF) and the growth factor (PGDF BB) in LP9/ TERT 1 cells (Figure 6B). Trends in diminishing levels of VEGF were also observed, although not statistically significant.

DISCUSSION

Gene expression analysis has been used for the classification of soluble toxicants in rodent and human cells *in vitro*. Models of

TABLE 2. TOP 10 GENES AFFECTED BY CROCIDOLITE ASBESTOS AT 8 AND 24 H IN LP9/TERT 1 HUMAN MESOTHELIAL CELLS

Concentration	Low ($15\ \mu m^2/cm^2$)		High ($75\ \mu m^2/cm^2$)
Time	8 h	24 h	8 h
	Fold Change		
Up-regulated			
Activating transcription factor 3 (*ATF3*)	9	9	27
Prostaglandin-endoperoxide synthase 2 (PTGS2)	7	8	16
Superoxide Dismutase 2 (SOD2)	6	6	2
Chemokine (C-X-C motif) ligand 3 (CXCL3)	4	NC	16
FBJ murine osteosarcoma viral oncogene homolog B (FOSB)	4	NC	NC
Tissue factor pathway inhibitor 2 (TFPI2)	4	14	11
Pyruvate dehydrogenase kinase, isozyme 4 (PDK4)	3	9	15
Chemokine (C-X-C motif) ligand 2 (CXCL2)	3	NC	NC
Angiopoietin-like 4 (ANGPLT4)	3	NC	NC
Kruppel-like factor 4 (gut) (KLF4)	3	NC	NC
Interleukin 8 C-terminal variant, 211506 s t (IL8)	NC	8	12
Interleukin 1 receptor-like 1 (IL1R1)	NC	6	11
Nuclear receptor subfamily 4 (NR4A2)	NC	NC	11
Solute carrier family 7 (SLC7A2)	NC	6	10
Pleckstrin homology-like domain (PHLDA1)	NC	7	NC
Interleukin 8 (IL8)	NC	6	NC
Down-regulated			
Inhibitor of DNA binding 3 (ID3)	NC	NC	5
Inhibitor of DNA binding 1 (ID1)	NC	NC	3
Cytochrome P450, family 24 (CYP24A1)	NC	NC	3
Basic helix-loop-helix domain (BHLHB3)	NC	NC	3
SMAD family member 6 (SMAD6)	NC	NC	3
S-phase kinase associated protein 2 (SKP2)	NC	NC	3
Cadherin 10, type 2 (CDH10)	NC	NC	3
START domain containing 5 (STARD5)	NC	NC	3
211042 x at	NC	NC	2
Interferon-induced protein with tetratricopeptide (IFIT1)	NC	NC	2
Oxytocin receptor (OXTR)	NC	6	NC
Transcribed locus	NC	5	NC
Chromosome 5 open reading frame (C5orf13)	NC	5	NC
Cytochrome P450, family 24 (CYP24A1)	NC	4	NC
Chromosome 21 open reading frame (C21orf7)	NC	3	NC
KIAA1199	NC	3	NC
Methyltransferase like 7A (METTL7A)	NC	3	NC
PDZ domain containing RING finger 3 (PDZRN3)	NC	3	NC
Periplakin (PPL)	NC	3	NC
Phospholipase-C-like 1 (PLCL1)	NC	3	NC

Definition of abbreviation: NC, no significant ($P \leq 0.05$) change $>$ 2-fold from control.

transcript profiling for discrimination of toxic and nontoxic compounds in liver and other organs have also been developed in rodents (18), confirming the hypothesis that predictive modeling for classification of toxic agents and carcinogens is feasible. Here we used toxicogenomic approaches in human mesothelial cells, a cell type exquisitely sensitive to asbestos (19) and human contact inhibited ovarian epithelial cells, a cell type not linked to carcinogenesis by asbestos, to determine whether the magnitude of altered gene expression by insoluble particulates correlated with their toxicity to cells and documented pathogenicity in humans. Although a recent study has examined gene expression profiles comparatively in crocidolite asbestos exposed human lung adenocarcinoma (A549) and SV40 immortalized bronchial (BEAS 2B) or pleural mesothelial cell lines (MET5A) by cluster analysis (20), our studies are the first to examine gene expression changes by asbestos in comparison to other well characterized particles in a human cell line that exhibits features of normal mesothelial cells (5). Although strict comparisons between cell types are not justified because SV40 Tag was used to immortalize the IOSE ovarian epithelial cell line (6), and SV40 infection is known to decrease sensitivity of human mesothelial cell lines to toxicity by asbestos (21), our studies suggest that the increased numbers of gene expression alterations observed in LP9/TERT 1 human mesothelial cells reflect elevated sensitivity of this cell type to asbestos. NYU474 human mesothelial cells were more resistant that LP9/TERT 1 cells to asbestos toxicity, permitting us to perform QRT PCR studies at both concentrations of asbestos at 24 hours. These results confirmed common dose related patterns of gene expression in mesothelial cells versus ovarian epithelial (IOSE) cells.

It is generally recognized that geometry and length and width (i.e., aspect ratio) of durable fibers such as amphibole asbestos types (crocidolite, amosite) are important properties determining toxicity, transforming potential, and carcinogeniciy in rodents and humans (13, 22, 23). Since talc can occur in various geometries (nonfibrous and fibrous) and can be contaminated with other minerals, including amphiboles, in some mining deposits (reviewed in Ref. 24), we used a well characterized, nonfibrous talc sample here to allow evaluation of a particle not causing mesotheliomas or pleural sarcomas in rodents (23). Moreover, nonfibrous talc is regarded as noncarcinogenic in humans (25). Since talc is a magnesium silicate, and Mg^{2+} may interact with negatively charged molecules on the cell surface to

TABLE 3. GENES UP REGULATED BY NONFIBROUS TALC IN LP9/TERT 1 HUMAN MESOTHELIAL CELLS

Gene	Fold Increase
8 h Low (15 $\mu m^2/cm^2$)	
Activating transcription factor 3 (*ATF3*)	3
8 h High (75 $\mu m^2/cm^2$)	
Activating transcription factor 3 (*ATF3*)	13
Inhibin, beta A (INHBA)	9
Chemokine (C-X-C motif) ligand 3 (CXCL3)	7
Superoxide dismutase 2 (SOD2)	7
Interleukin 8 C-terminal variant, 211506 s t (IL8)	6
Prostaglandin-endoperoxide synthase 2 (PTGS2)	5
Interleukin 8 (IL8)	5
FBJ murine osteosarcoma viral oncogene homolog B (FOSB)	5
Tumor necrosis factor alpha-induced protein 6 (TNFAIP6)	4
Tissue factor pathway inhibitor 2 (TFPI2)	4
Chemokine (C-X-C motif) ligand 2 (CXCL2)	3
Intercellular adhesion molecule 4 (CICAM4)	3
ChaC, cation transport regulator homolog 1 (ChaC 1)	3
Nuclear receptor subfamily 4, group A, member 3 (NR4A3)	3
Pleckstrin homology-like domain, family A, member 1 (PHLDA1)	3
Interleukin 6 (IL-6)	3
Phorbol -12-myristate-13-acetate-induced protein 1 (PMA1P1)	3
Oxidized low density lipoprotein (lectin-like) receptor 1 (OLR1)	3
Chemokine (C-C motif) ligand 20 (CCL20)	3
v-maf musculoaponeurotic fibrosarcoma oncogene homolog F	3
Interleukin 1, alpha (IL-1α)	2
Tumor necrosis factor-α induced protein 3 (TNFA1P3)	2
Interleukin 1 receptor-like 1 (IL1RL1)	2
Angiopoieten-like 4 (ANGPLT4)	2
Kruppel-like factor 4 (KLF4)	2
GTP binding protein overexpressed in skeletal muscle (GEM)	2
Pentraxin-related gene, rapidly induced by IL-1 beta (PTX3)	2
Interleukin 1 beta (IL-1β)	2
HSPB (heat shock 27 kD) associated protein 1 (HSPBAP1)	2
Kynureninase (KYNU)	2

disturb cell homeostasis (reviewed in Ref. 26), this may explain the few mRNA expression increases that were observed initially with talc at 8 hours. However, these changes were not observed at 24 hours, suggesting that human mesothelial cells adapt to or undergo repair after exposure to this mineral.

Our gene profiling data here and in inhalation studies using chrysotile asbestos (14) also support the concept that fine TiO_2 is nontoxic and nonpathogenic to mesothelial or other cell

TABLE 4: GENES UPREGULATED BY CROCIDOLITE ASBESTOS IN IOSE HUMAN OVARIAN CELLS

Gene	Fold increase
8 h High (75 $\mu m^2/cm^2$)	
Nuclear receptor subfamily 4 (NR4A2)	4
Chemokine (C-X-C motif) ligand 2 (MIP2)	2
24 h High (75 $\mu m^2/cm^2$)	
Nuclear receptor subfamily 4 (NR4A2)	4
DNA-damage-inducible transcript 3 (DDIT3)	3
Stromal cell-derived factor 2-like 1(SDF2L1)	3
Heat shock 70 kD protein 1A (HSPA1A)	3
DnaJ (Hsp40) homolog, subfamily C (DNAJC3)	2
Paraspeckle component 1	2
Heat shock 70 kD protein 1B (HSPA1B)	2
Homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member (HERPUD1)	2
Serum/glucocorticoid regulated kinase family, member 3 (SKG3)	2
DnaJ (Hsp40) homolog, subfamily B, member 9 (DNAJB9)	2
Arginine-rich, mutated in early stage tumors (ARMET)	2
Syntaxin 1A (brain) (STX1A)	2
Heat shock 70 kD protein 5 (HSPA5)	2
ADAM metallopeptidase with thrombospondin type 1 motif	2
Heat shock protein 90kDa beta (Grp94), member 1 (HSP90B1)	2

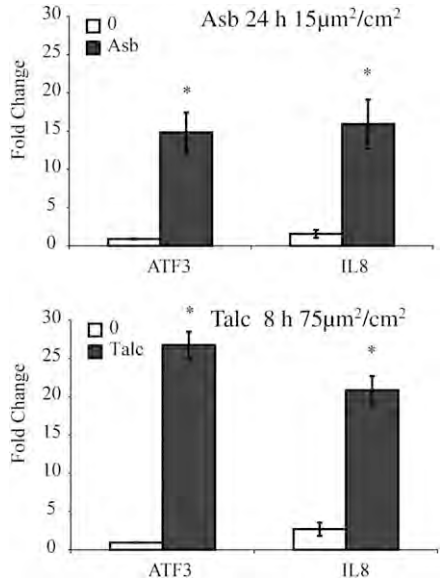

Figure 4. QRT PCR confirms significant increases in *ATF3* and *IL8* expression by crocidolite asbestos at low concentrations and non fibrous talc at high concentrations in LP9/TERT 1 mesothelial cells. *$P <$ 0.05 as compared to untreated (0) groups.

types. Likewise, in the rat, inhalation of fine TiO_2 (defined as particles $>$ 0.1 μm in diameter), in contrast to ultrafine (particles $<$ 0.1 μm in diameter) does not give rise to predictive markers of toxicity, inflammation, pulmonary fibrosis, or oxi dative stress, as indicated by elevated levels of Mn containing superoxide dismutase (*SOD2*) in cells from bronchopulmonary lavage (27). The increased reactivity and toxicity of ultrafine particles as compared with larger fine or coarse particles have also been confirmed in a number of *in vitro* and *in vivo* experi ments and is often attributed to their increased surface area and/ or ability to penetrate lung cells.

Our studies reveal a number of novel genes induced by asbestos in LP9/TERT 1 cells. As previously described in a lung epithelial cell line (C10) or mouse lungs after inhalation of crocidolite asbestos (28), increases in expression of the early response gene, *FOSB*, that encodes a dimer of the activator protein 1 transcription factor, were seen. Increases in expression of several other genes linked to cell signaling proteins and transcription factor activation were observed in asbestos exposed cells, including *NR4A2* and *PDK4*. A novel gene up regulated at all time points and concentrations of asbestos or talc in human mesothelial cells was activating transcription factor 3 (*ATF3*), a member of the cAMP re sponsive element binding (CREB) transcription factor family that encodes two different isoforms leading to repression or activation of genes. Silencing of *ATF3* in the present study by siRNA significantly altered expression of a number of asbestos induced inflammatory cytokines and growth factors docu mented in malignant mesotheliomas (29, 30). In support of our results here, other studies using *ATF3* deficient mice and *in vitro* approaches have shown that *ATF3* is a negative regulator of pulmonary inflammation, eosinophilia, and airway responsiveness (31). Moreover, *ATF3* also negatively regulates IL 6 gene transcription in an NF κB model of up regulation using melanoma cells (32). In addition, trends in production of VEGF, a known important angiogenic peptide and independent prognostic factor in human mesotheliomas (33), were observed. We have recently shown that an extracellular signal related

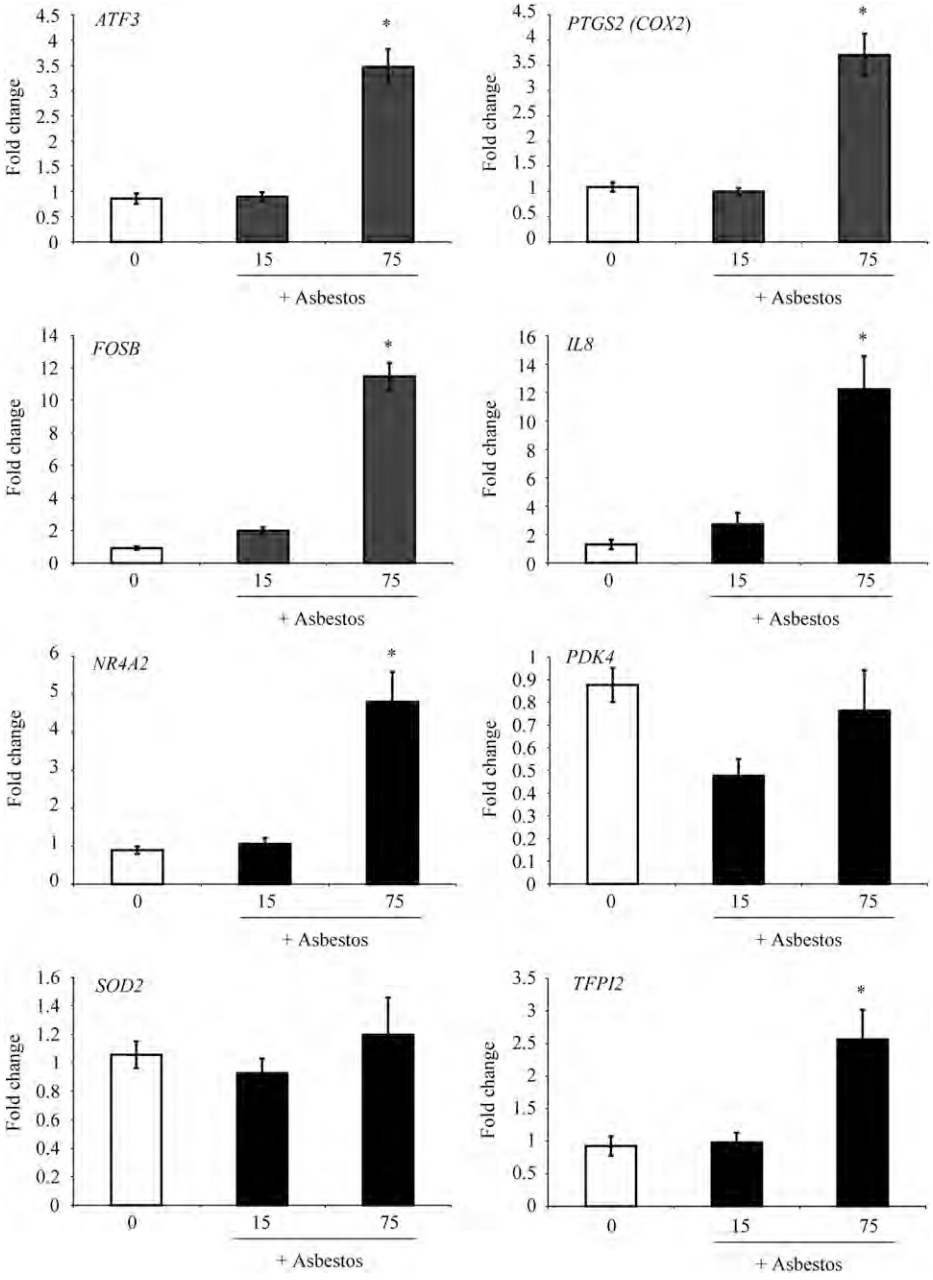

Figure 5. QRT PCR confirms that human primary pleural mesothelial cells (NYU474) show similar patterns of asbestos induced gene expression when compared with LP9/TERT 1 mesothelial cells. NYU474 cells were exposed to crocidolite asbestos (15 or 75 $\mu m^2/cm^2$) for 24 hours and cDNA was used for QRT PCR. *$P \leq 0.05$ as compared with untreated cells (0).

CREB pathway in C10 lung epithelial cells modulates apoptosis after asbestos exposure (34), and recent studies are focusing on the effects of silencing *CREB* or *ATF3* on other functional and phenotypic changes in human mesothelial and mesothelioma cells (A. Shukla and colleagues, unpublished data).

Several other genes up regulated by talc at 8 hours or affected by asbestos at both 8 and 24 hours may be important in repair from mineral induced responses. For example, *SOD2*, (Mn containing superoxide dismutase) is an antioxidant protein occurring in the mitochondria, a target cell organ of asbestos induced apoptosis (35). *PTGS2* (prostaglandin endoperoxide syntase or cyclooxyge nase) is a key enzyme in prostenoid biosynthesis associated with modulation of mitogenesis and inflammation. More recently, this pathway has been explored after interaction of ultrafine particles with alveolar macrophages (9). *ANG PTL4* (angiopoietin 4) encodes a serum hormone directly involved in regulating glucose homeostasis and lipid metabolism and is an apoptosis survival factor for vascular endothelial cells. The up regulation of angiopoietin 4 is also thought to play a role in inhibition of tumor cell motility and metastasis. *KLF4* (Kruppel like factor 4) is a negative regulator of cell proliferation and can be a positive or negative modulator of DNA transcription.

Increased expression of genes encoding different cytokines/chemokines (i.e., *IL8*) and their receptors or ligands (e.g., IL 8 C terminal variant, *IL1R1*, *CXCL2* or *MIP2*, *CXCL3*, and *TFP12*) by asbestos or talc suggests that the mesothelial cell also may play a role in chemotaxis, inflammation, and blood coagulation. A number of gene expression changes by asbestos also support the hypothesis that this fibrous mineral affects calcium depen dent processes including related protein kinase cascades, cell adhesion, and protein/lipid metabolism (Table 2). Although numbers of changes were more modest in IOSE cells, with the exception of *NR4A2* and *CXCL2*, a unique subset of genes was induced by asbestos in this cell type (Table 4).

Results of work here suggest that transcriptional profiling can be used to reveal molecular events by mineral dusts that are

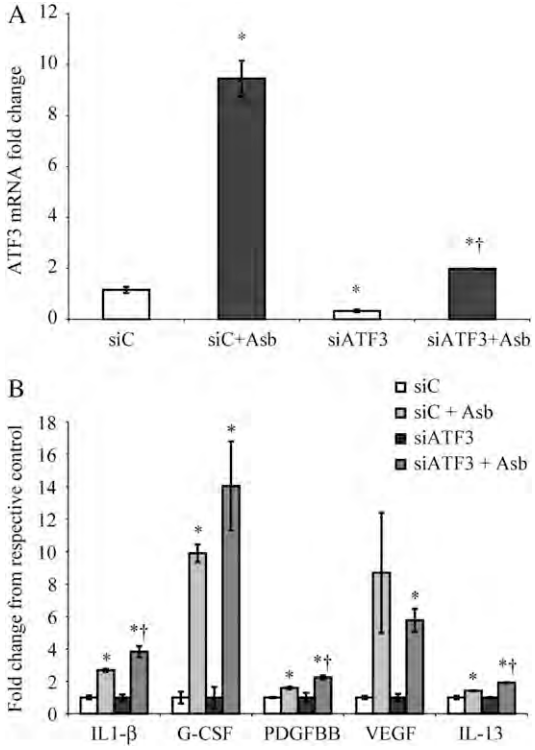

Figure 6. *ATF3* inhibition using siRNA approaches alters asbestos induced production of inflammatory cytokines and growth factors. (*A*) LP9/TERT 1 cells transfected with siATF3 show significant inhibition of *ATF3* mRNA levels (untreated control [siC] versus siATF3 and asbestos treated [siC Asb versus siATF3 Asb] groups). $*P \leq 0.05$ as compared with siC; $\dagger P \leq 0.05$ as compared with siC Asb group. (*B*) siATF3 altered asbestos induced cytokine levels as detected in medium at 24 hours using Bio Plex analyses. $*P \leq 0.05$ as compared with control groups (siC and siATF3), respectively; $\dagger P \leq 0.05$ as compared with asbestos exposed scrambled control group (siC).

predictive of their pathogenicity in mesothelioma. Moreover, they reveal early and novel gene responses, including calcium dependent transcription factors and antioxidant enzymes that may be pursued for their functional significance using RNA silencing or other approaches.

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Exhibit 94

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Pycnogenol® reduces Talc-induced Neoplastic Transformation in Human Ovarian Cell Cultures

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Talc and poor diet have been suggested to increase the risk of developing ovarian cancer; which can be reduced by a diet rich in fruit and vegetables. Talc is ubiquitous despite concern about its safety, role as a possible carcinogen and known ability to cause irritation and inflammation. It was recently shown that Pycnogenol® (Pyc; a proprietary mixture of water-soluble bioflavonoids extracted from French maritime pine bark) was selectively toxic to established malignant ovarian germ cells. This study investigated talc-induced carcinogenesis and Pyc-induced chemoprevention. Normal human epithelial and granulosa ovarian cell lines and polymorphonuclear neutrophils (PMN) were treated with talc, or pretreated with Pyc then talc. Cell viability, reactive oxygen species (ROS) generation and neoplastic transformation by soft agar assay were measured. Talc increased proliferation, induced neoplastic transformation and increased ROS generation time-dependently in the ovarian cells and dose-dependently in the PMN. Pretreatment with Pyc inhibited the talc-induced increase in proliferation, decreased the number of transformed colonies and decreased the ROS generation in the ovarian cells. The data suggest that talc may contribute to ovarian neoplastic transformation and Pyc reduced the talc-induced transformation. Taken together, Pyc may prove to be a potent chemopreventative agent against ovarian carcinogenesis. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: ovarian cancer; talc; Pycnogenol®; human neutrophils.

INTRODUCTION

Ovarian cancer is the sixth most commonly occurring cancer and ranks fifth in cancer deaths among women, accounting for more deaths than any other cancer of the female reproductive system. Epidemiological studies have suggested that diet, talc, industrial pollutants, smoking, asbestos and infectious agents may increase the risk of developing ovarian cancer (American Cancer Society, 2000) and may do so by causing localized inflammation (Ness and Cottreau, 1999). Specifically, talc exposure has been cited as a risk factor because of its similarity to asbestos (Cramer *et al.*, 1999).

Talc is a layered magnesium silicate [$Mg_3Si_4O_{10}(OH)_2$]. It is used in cosmetics (as the primary ingredient in talcum powder), pharmaceuticals (as an excipient in tablets) and in many other industrial applications (Bremmell and Addai-Mensah, 2005). Talc is used medically to induce pleurodesis because of its known ability to cause irritation and inflammation (Holthouse and Chleboun, 2001). Animal studies showed a systemic migration of talc particles to various organs despite route of entry (Henderson *et al.*, 1986; Werebe *et al.*, 1999). Exposure of rat ovaries to talc leads to cyst formation (Hamilton *et al.*, 1984). Talc was also shown to cause superoxide anion generation and release from murine macrophages (Van Dyke *et al.*, 2003). Thus controversy continues to surround the topic of talc, its safety (Janssen, 2004) and its role as a possible carcinogen (Cramer *et al.*, 1999; Wong *et al.*, 1999).

Lifestyle factors are important in the etiology of ovarian cancer and current evidence suggests the risk can be reduced by eating a diet rich in fruit and vegetables, among other lifestyle choices (Hanna and Adams, 2006). For the past 20 years, researchers have proposed that nutritional factors play one of the most important roles in the etiology of human cancer. It is estimated that 35% (range 10–70%) of all cancers are diet related and that consumption of certain fruits and vegetables is inversely associated with the incidence of specific forms of cancer. Past research has indicated that a large number of bioactive components, which proved to be protective on different stages of cancer formation, have been identified in nutrients that are of plant origin (Knasmuller and Verhagen, 2002).

Pycnogenol® (Pyc) is a proprietary mixture of water-soluble bioflavonoids extracted from the bark of French maritime pine (*Pinus maritima* Aiton; currently known as *Pinus pinaster* Aiton). The main constituents of Pyc are phenolic compounds, broadly divided into monomers (catechin, epicatechin and taxifolin) and condensed flavonoids (classified as procyanidins and proanthocyanidins). Pyc is known to possess potent antioxidant activity, it not only scavenges the free radicals but it also enhances the endogenous antioxidant systems (Nelson *et al.*, 1998; Wei *et al.*, 1997). Pyc has also been shown to selectively induce apoptosis in breast cancer cells (Huynh and Teel, 2000) and induce differentiation and apoptosis in human promyeloid leukemia cells (Huang *et al.*, 2005). It was previously

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shown that Pyc selectively induced cell death in established malignant ovarian germ cells *in vitro* (Buz'Zard and Lau, 2004). This study now reports that Pyc prevents talc-induced neoplastic transformation of normal ovarian cells, *in vitro*.

MATERIALS AND METHODS

Reagents and chemicals. Pycnogenol® was supplied by Horphag Research (Geneva, Switzerland). Talc, crystal violet, Giemsa stain, RPMI-1640 medium and other miscellaneous chemicals were purchased from Sigma (St Louis, MO). Polymorphoprep™ was purchased from Greiner Bio-One, Inc. (Longwood, FL). Dulbecco's modification of Eagle's Medium (DMEM), Ham's F-12 medium and penicillin–streptomycin (P-S) were purchased from Cellgro (Herndon, VA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). The CellTiter 96® AQueous One Solution Cell Proliferation Assay was purchased from Promega (Madison, WI). High strength analytical grade agarose was purchased from Bio-Rad (Hercules, CA). Ionagar No. 2 was purchased from Oxoid (London, UK). 5-(and-6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) was purchased from Molecular Probes (Carlsbad, CA).

Water soluble extraction of Pycnogenol®. Pyc was incubated at 56 °C for 5 h in double distilled water, allowed to cool to room temperature and filtered using a Steriflip® Vacuum Filtration System (0.22 µm Durapore PVDF membrane; Millipore Corporation, Bedford, MA).

Cell culture and treatments. Two cell cultures of human origin were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. OSE2a (immortalized normal ovarian epithelial) and GC1a (immortalized normal ovarian granulosa) cell cultures were donated by Dr Hitoshi Okamura at Kumamoto University, Japan (Okamura *et al.*, 2003). The cell lines were maintained in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 10% FBS and 100 IU/mL P-S. In preparation for either talc or Pyc + talc treatments, each cell line was seeded (1×10^5 cells/ml) and grown to 80% confluence, unless otherwise specified. Cells were incubated with 0–500 µg/mL talc from 24 to 120 h; or 0–500 µg/mL Pyc for 24 h followed by 5 µg/mL talc for 24 or 72 h.

Neutrophil isolation and culture. Peripheral blood polymorphonuclear neutrophils (PMN) and monocytes were obtained from heparinized venous blood from healthy volunteers (protocol approved by Loma Linda University Institutional Review Board for Human Studies) and isolated by Polymorphoprep™ density gradient centrifugation followed by the hypotonic lysis of erythrocytes. The purity of PMNs was determined by Giemsa staining as greater than 95%. Purified cells were suspended at 5×10^5 cells/mL in RPMI-1640 containing 2 mM L-glutamine, 1 mM sodium pyruvate, supplemented with 10% FBS and 100 IU/mL P-S; and treated with varying concentrations of talc for 24 or 72 h. ROS generation was detected as detailed below.

Cell viability assay. The CellTiter 96® AQueous One Solution Cell Proliferation Assay was used to measure cell viability (Buz'Zard and Lau, 2004). The MTS [3-(4,5-dimethylthiazolyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] solution was used according to manufacturer's instructions. The absorbance was read at 490 nm using a model 3550 Microplate Reader (Bio-Rad). The percent cell viability was calculated as the absorbance of the treated cells divided by the absorbance of the untreated-control cells multiplied by 100.

Neoplastic transformation assay. A characteristic of cancer cells is their ability to grow and to divide when held in suspension without attachment or with minimal attachment to a rigid surface (Leung *et al.*, 2004). Thus, growth in soft agar demonstrates *in vitro* transformation of cells to their neoplastic counterparts (Morales *et al.*, 2003). After 72 h of incubation in the presence of talc; or in the presence of 0–500 µg/mL Pyc for 24 h followed by 5 µg/mL talc for 72 h, cells were collected, washed and suspended in 0.35% agarose at 5000 cells/well and layered on top of a base of 0.5% agar. The plates were incubated at 37 °C in a humidified incubator for 14 days. The cells were stained with 0.005% crystal violet and colonies were counted using an inverted microscope (Cory *et al.*, 1987).

Reactive oxygen species (ROS) detection. Carboxy-H_2DCFDA is a non-fluorescent dye that permeates the cells where it is deacetylated by viable cells to 2′,7′-dichlorofluorescin (DCFH), which is then oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF) by endogenous hydrogen peroxide (H_2O_2) (Wan *et al.*, 1993). The cells were seeded in Optilux™ 96-well plates (BD Falcon, Bedford, MA) and treated with 0 to 500 µg/mL Pyc for 24 h. H_2O_2 (100 µM) was used as a positive control. Carboxy-H_2DCFDA (5 µM) was added and incubated for 1 h. The fluorescence intensity (excitation 485 nm/emission 530 nm) was measured as arbitrary fluorescent units (AFU) using a model 7600 Microplate Fluorometer (Cambridge Technology, Inc., Watertown, MA). The percent AFU (a.k.a. % ROS generation) was calculated as the 'treated cell-AFU' divided by the 'untreated cell-AFU' multiplied by 100. Immediately following the fluorescence detection, the fluorescence intensity was normalized by the cell viability assay.

Statistical analysis. Data were reported as mean ± SE. Statistical analysis was performed with the Student's paired *t*-test.

RESULTS

All experiments were performed a minimum of three times with reproducible results.

Effect of talc on cell viability of normal ovarian cells

Talc caused a bell-shaped curve response in OSE2a cells, with a statistically significant increase seen at 5 µg/mL (24 h) and a statistically significant decrease at 200 µg/mL (72 h) and 500 µg/mL (24 and 72 h) (Fig. 1).

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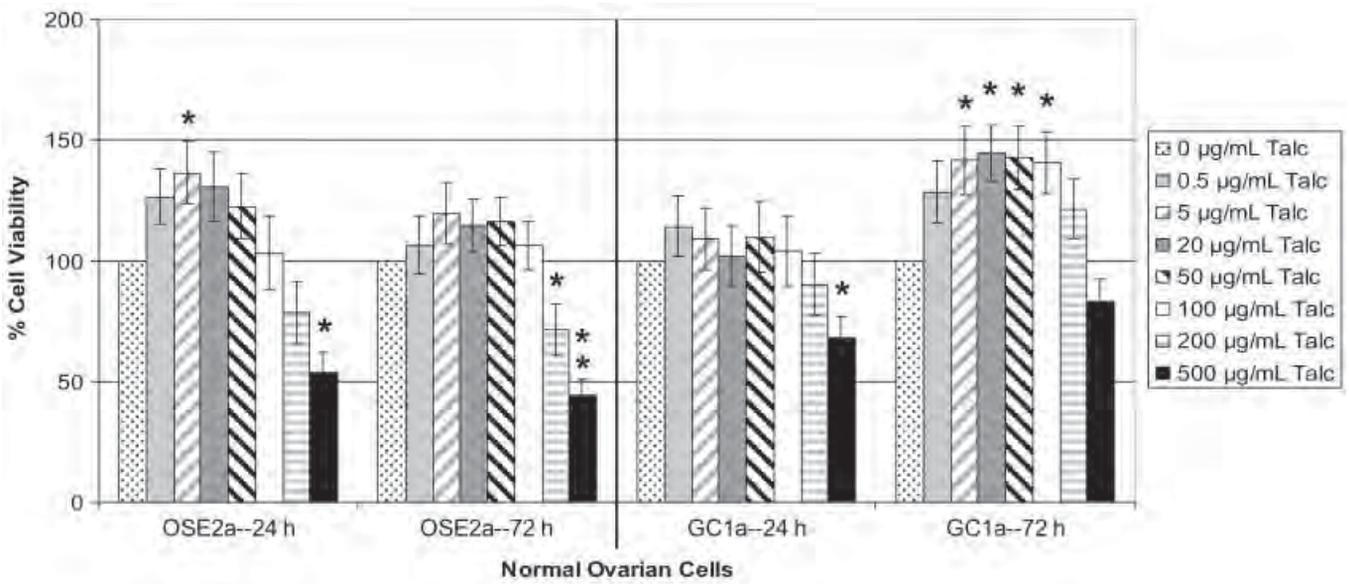

Figure 1. Effect of talc on the cell viability of ovarian cells. Normal ovarian epithelial (OSE2a) and normal ovarian stromal (GC1a) cells were treated with various concentrations of talc for 24 and 72 h. Cell viability was measured by the MTS assay and the percent cell viability was calculated as the absorbance of the treated cell divided by the absorbance of the untreated-control cells multiplied by 100. Each data point represents mean ± SE of five determinations. Statistical significance was determined by the Student's paired *t*-test. * $p < 0.05$, ** $p < 0.01$ comparing the treatment with the respective untreated control.

Also seen in Fig. 1, talc caused a bell-shaped curve response in GC1a cells, with a statistically significant increase seen at 5, 20, 50 and 100 µg/mL (72 h) and a statistically significant decrease at 500 µg/mL (24 h).

Effect of talc on neoplastic transformation of normal ovarian cells

Since the ability to grow suspended in soft agar is a characteristic of cells being transformed to their neoplastic counterparts (Leung *et al.*, 2004; Morales *et al.*, 2003), the study determined whether talc would be able to induce such a transformation. As shown in Fig. 2, talc caused a statistically significant increase in the number of transformed colonies in the OSE2a cells at 5 and 20 µg/mL talc and in the GC1a cells at 5, 20 and 100 µg/mL talc, compared with the untreated control. An exception was seen in the 100 µg/mL talc treatment in the OSE2a cells in which the number of transformed colonies was reduced significantly.

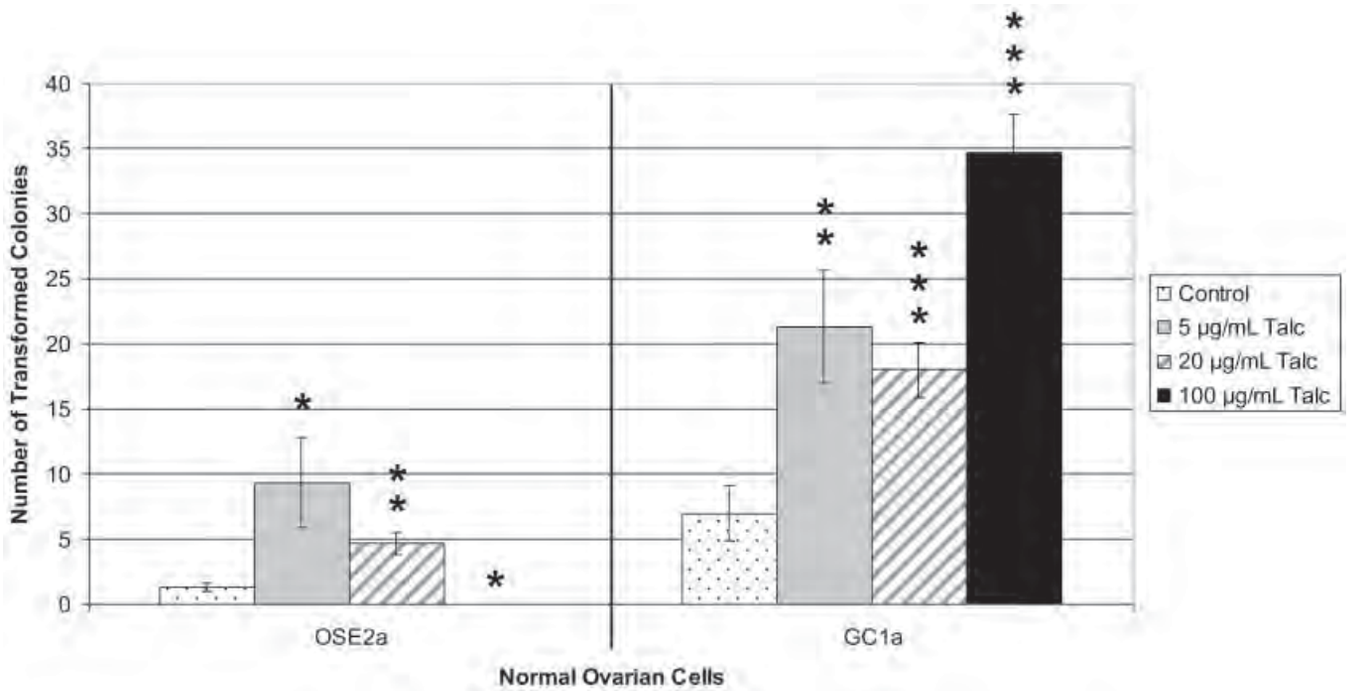

Figure 2. Neoplastic transformation of ovarian cells by talc. Normal ovarian epithelial (OSE2a) and normal ovarian stromal (GC1a) cells were incubated with various concentrations of talc for 72 h, collected, washed, seeded in soft agar suspension and grown for 14 days before colonies were counted. Each data point represents mean ± SE of three determinations. Statistical significance was determined by the Student's paired *t*-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ comparing the treatment with the respective untreated control (0 µg/mL talc).

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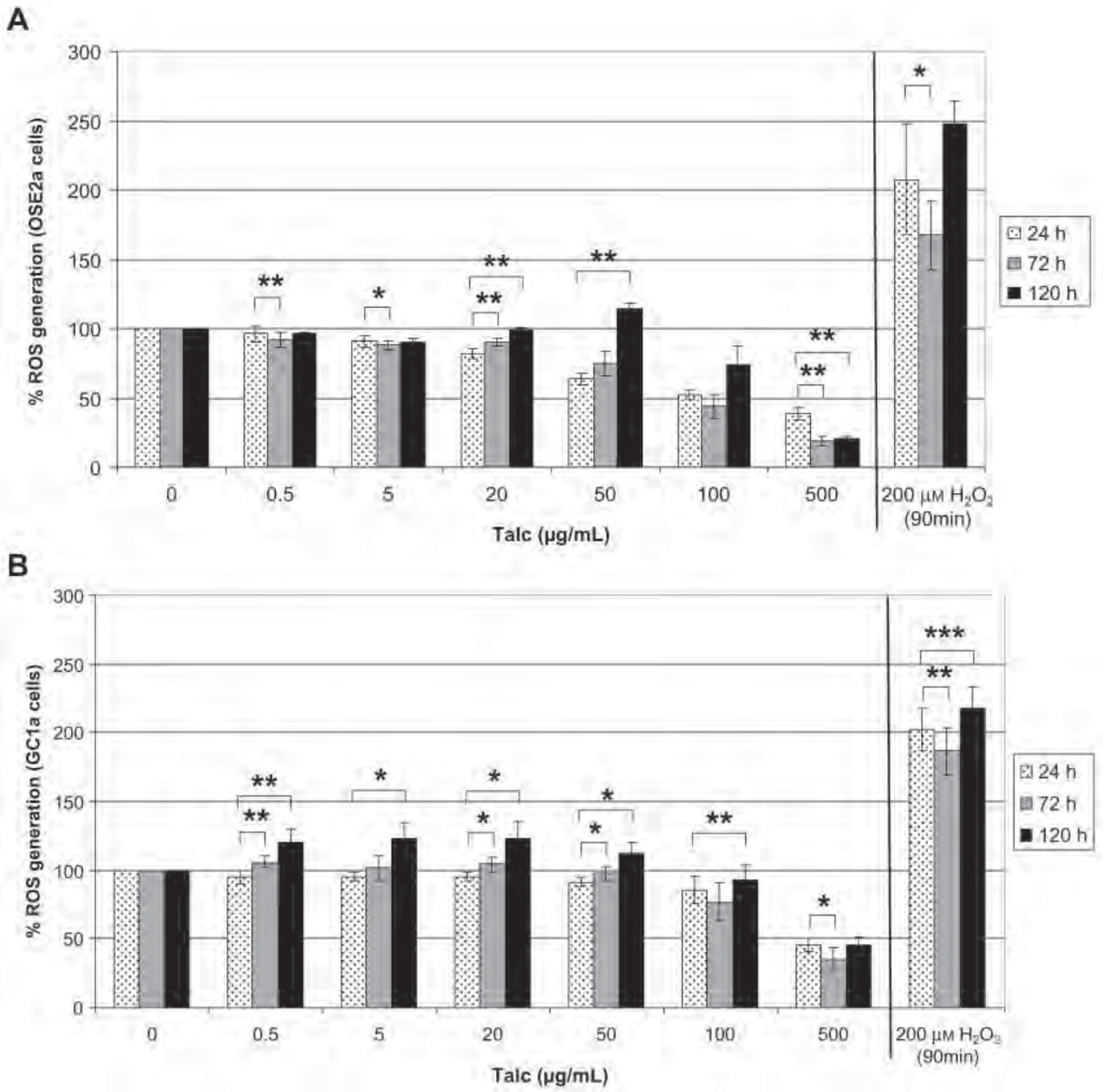

Figure 3. ROS generation of ovarian cells in response to talc treatments. Normal ovarian epithelial (OSE2a) and normal ovarian stromal (GC1a) cells were treated with various concentrations of talc for 24, 72 and 120 h and H_2O_2 during the last 90 min of each respective time point. H_2O_2 was used as a positive control for this assay. Fluorescence intensity were measured as arbitrary fluorescent units (AFU) at ex 485 nm/em 530 nm and normalized with the cell viability assay. Percent AFU (a.k.a. % ROS generation) was calculated as the average AFU of the treated cell divided by the average AFU of the untreated-control cells multiplied by 100. (A) ROS generation in OSE2a cells in response to talc treatments. (B) ROS generation in GC1a cells in response to talc treatments. Each data point represents mean ± SE of three determinations. Statistical significance was determined by the Student's paired *t*-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ comparing the treatment with the respective untreated control (as demonstrated by the horizontal brackets).

Effect of talc on ROS generation in normal ovarian cells

Talc caused an initial dose-dependent decrease in ROS generation (24 h) which increased with time in OSE2a cells (Fig. 3A). However, as time increased, ROS generation rebounded and increased compared with the values at 24 h. A statistically significant increase was seen at 20 μg/mL (72 and 120 h) and 50 μg/mL (120 h). Talc also caused an initial dose-dependent decrease in ROS generation (24 h) in GC1a cells (Fig. 3B), but ROS generation increased with time in the talc treated cells. A statistically significant increase was seen with 0.5, 20 and 50 μg/mL (72 and 120 h), as well as 5 and 100 μg/mL (120 h) compared with the respective 24 h value.

Effect of talc on ROS generation in PMN

Since oxidative stress is often a component of the tumor microenvironment (Valko *et al.*, 2004), the study tested whether talc was capable of inducing ROS generation

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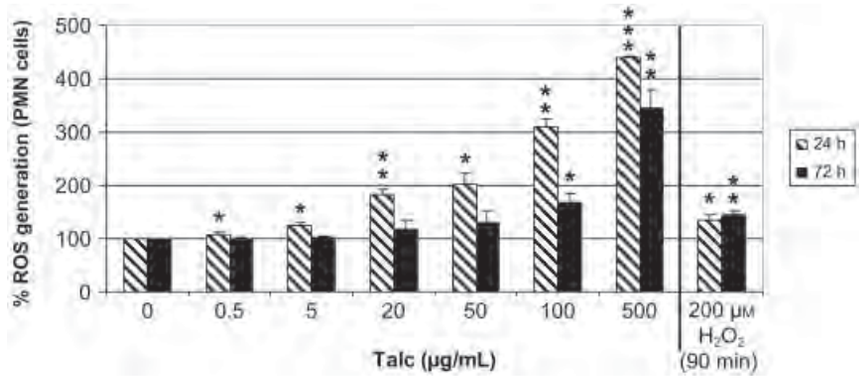

Figure 4. ROS generation of polymorphonuclear neutrophils (PMN) in response to talc treatments. PMNs were treated with various concentrations of talc for 24 and 72 h and H_2O_2 during the last 90 min of each respective time point. H_2O_2 was used as a positive control for this assay. Fluorescence intensity were measured as arbitrary fluorescent units (AFU) at ex 485 nm/em 530 nm and normalized with the cell viability assay. Percent AFU (a.k.a. % ROS generation) was calculated as the average AFU of the treated cell divided by the average AFU of the untreated-control cells multiplied by 100. ROS generation of PMNs in response to talc treatments. Each data point represents mean ± SE of three determinations. Statistical significance was determined by the Student's paired *t*-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ comparing the treatment with the respective untreated control.

in human PMNs. Talc caused a dose-dependent increase in ROS generation at both time points (Fig. 4). The increase was statistically significant at 0.5, 5, 20, 50 µg/mL (24 h) and 100 and 500 µg/mL (24 and 72 h). The maximum ROS generation was seen at 500 µg/mL and was increased over 4-fold at 24 h and 3.5-fold at 72 h, compared with the respective untreated cells.

Effect of pretreatment with Pyc on talc-induced cell viability changes in normal ovarian cells

Pretreatment with Pyc did not cause a statistically different change in cell viability in the OSE2a cells (Fig. 5A). Pretreatment with Pyc caused a general decrease in cell viability in the GC1a cells (Fig. 5B) compared with the respective untreated GC1a cells. One exception is that of a slight, but statistically significant, increase in cell viability at 100 µg/mL Pyc + 5 µg/mL talc (72 h) compared with the respective untreated GC1a cells (Fig. 5B).

Effect of pretreatment with Pyc on talc-induced neoplastic transformation of normal ovarian cells

Pretreatment with Pyc decreased the number of neoplastically transformed colonies induced by talc in

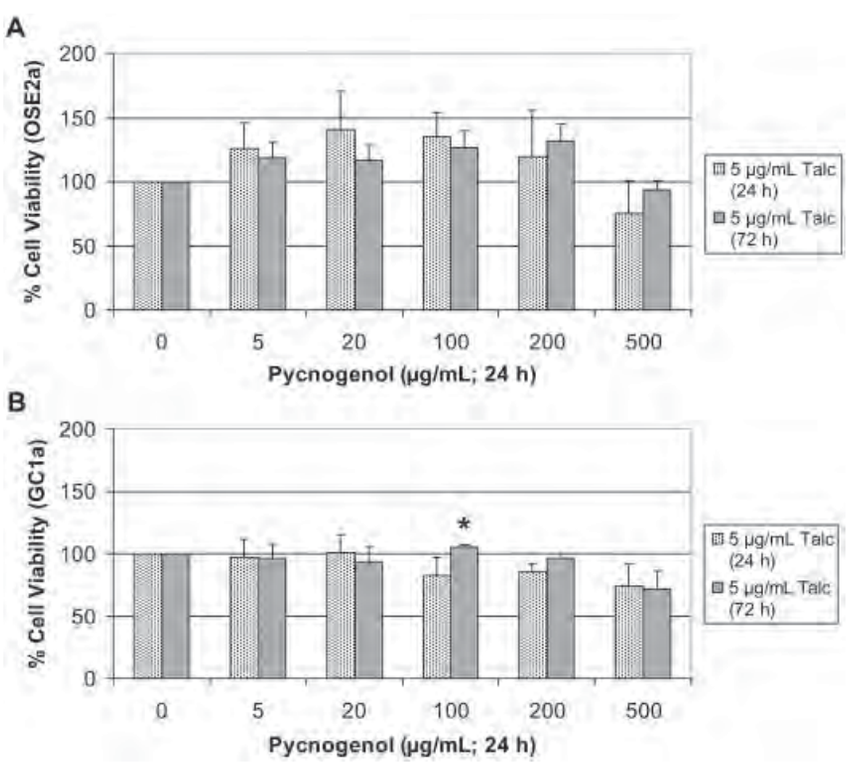

Figure 5. Effect of Pyc + talc treatments on the cell viability of ovarian cells. Normal ovarian epithelial (OSE2a) and stromal (GC1a) cells were treated with 0–500 µg/mL Pyc for 24 h followed by 5 µg/mL talc for 24 and 72 h. Cell viability was measured by the MTS assay and percent cell viability was calculated as the absorbance of the treated cell divided by the absorbance of the untreated-control cells multiplied by 100. (A) OSE2a cells. (B) GC1a cells. Each data represent mean ± SE of four determinations. Statistical significance was determined by the Student's paired *t*-test. * $p < 0.05$ comparing the treatment with the respective untreated control.

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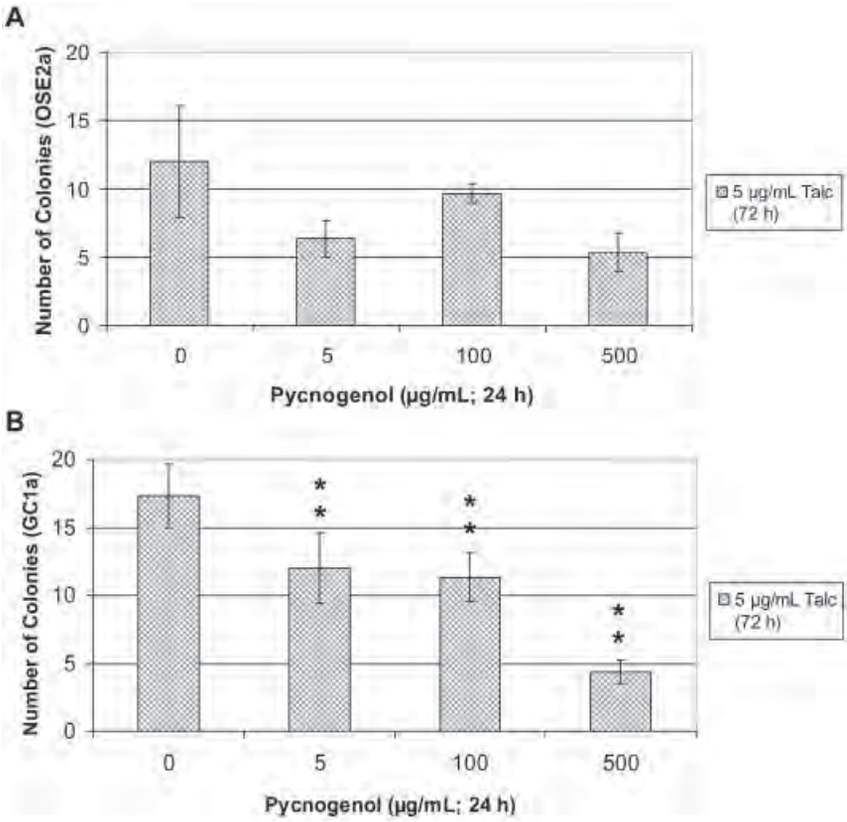

Figure 6. Pyc-induced protection against neoplastic transformation of ovarian cells by talc. Normal ovarian epithelial (OSE2a) and stromal (GC1a) cells were incubated with 0–500 µg/mL Pyc for 24 h followed by 5 µg/mL talc for 72 h, collected, washed, seeded in soft agar suspension and grown for 14 days before colonies were counted. (A) OSE2a cells. (B) GC1a cells. Each data represent mean ± SE of three determinations. Statistical significance was determined by the Student's paired *t*-test. ** $p < 0.01$ comparing the treatment with the respective control.

the OSE2a cells, but not in a statistically significant manner (Fig. 6A). Pretreatment with Pyc (5, 100 and 500 µg/mL; 24 h) caused a statistically significant decrease in the number of talc-induced neoplastically transformed colonies in the GC1a cells (Fig. 6B).

Effect of pretreatment with Pyc on talc-induced ROS generation in normal ovarian cells

Pretreatment with Pyc caused a statistically significant decrease in ROS generation at 5, 20, 50, 100 and 200 µg/mL Pyc + 5 µg/mL talc (24 h); and 500 µg/mL Pyc + 5 µg/mL talc (24 and 72 h) in the OSE2a cells (Fig. 7A). Pretreatment with Pyc caused a statistically significant decrease in ROS generation at 5, 20, 50, 200 and 500 µg/mL Pyc + 5 µg/mL talc (24 h) in the GC1a cells (Fig. 7B). Pretreatment with Pyc caused a statistically significant decrease in ROS generation at 5, 20, 50, 100, 200 and 500 µg/mL Pyc + 5 µg/mL talc (72 h) in the GC1a cells (Fig. 7B). The decrease seen at 100 µg/mL Pyc + 5 µg/mL talc (24 h) was not statistically significant (Fig. 7B).

DISCUSSION

Cancer development is a multi-step process comprising a series of cellular and molecular changes that are mediated by various endogenous and exogenous stimuli, such as aberrantly expressed ROS (Storz, 2005). Although ROS are a byproduct of endogenous biochemical processes, ROS (such as H_2O_2) at high concentrations or expressed in a chronic nature can damage cellular macromolecules and contribute to neoplastic transformation and tumor growth (Nicco *et al.*, 2005). A characteristic of neoplastically transformed cells is their ability to grow and to divide when held in suspension without attachment or with minimal attachment to a rigid surface (Leung *et al.*, 2004; Morales *et al.*, 2003). Our data show that talc not only increased cell viability (Fig. 1A), but also caused an increase in transformed cells in both the stromal and epithelial ovarian cells by their ability to grow, divide and form colonies while being suspended in soft agar (Fig. 2A).

It is known that substances that raise the intracellular level of H_2O_2 are able to trigger normal cell proliferation and abolish tumor cell proliferation (Ness and Cottreau, 1999; Nicco *et al.*, 2005). In normal cells, the basal level of H_2O_2 is low and its increase is initially associated with cell growth. H_2O_2 at high concentrations or expressed in a chronic nature in normal cells, can damage cellular macromolecules and contribute to neoplastic transformation and tumor growth (Nicco *et al.*, 2005). In this study, talc was shown to increase the ROS generation, after an initial suppression, in a time-dependent manner in the normal stromal cells (Fig. 3B) and less strongly in the normal epithelial cells (Fig. 3A).

Recent studies have expanded the concept that inflammation is a critical component of tumor progression. The neoplastic process (proliferation, survival and

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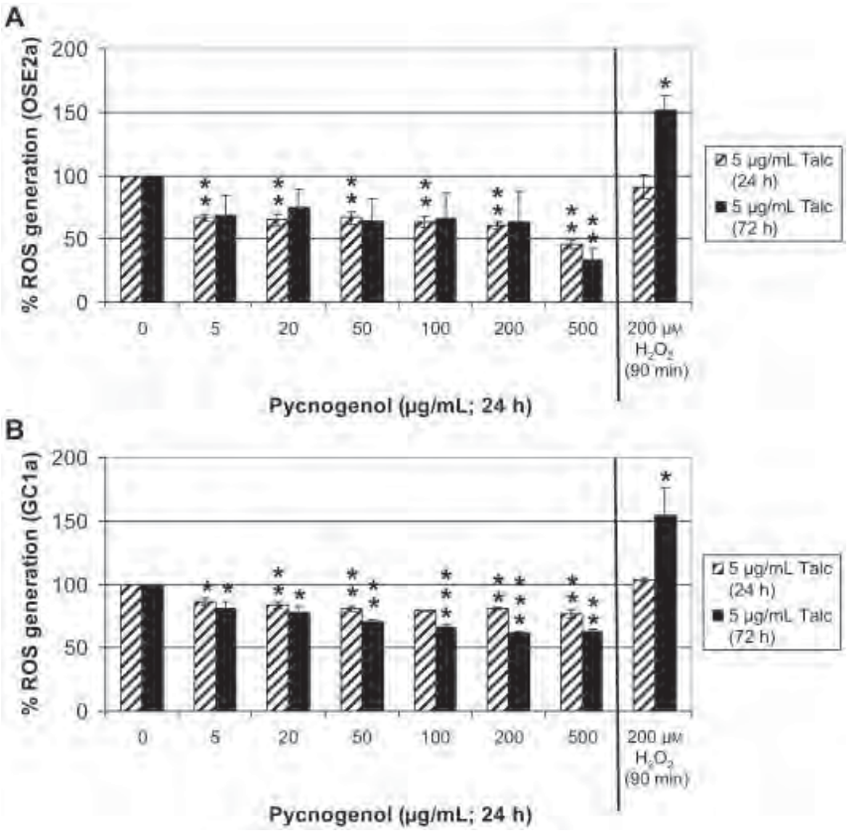

Figure 7. ROS generation of ovarian cells in response to Pyc + talc. Normal ovarian epithelial (OSE2a) and stromal (GC1a) cells were treated with 0–500 µg/mL Pyc for 24 h, followed by 5 µg/mL talc for 24 or 72 h and H_2O_2 (the last 90 min of each time point) as a positive control. Fluorescence intensity (AFU) was measured at ex 485 nm/em 530 nm and normalized by cell viability assay. The percent ROS generation was calculated as the average AFU of treated divided by AFU of untreated-control multiplied by 100. (A) OSE2a cells. (B) GC1a cells. Each data point represents mean ± SE of three determinations. Statistical significance was determined by the Student's paired *t*-test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ comparing the treatment with the respective untreated control.

migration) is linked with the tumor microenvironment and synchronized with inflammatory cells (Valko *et al.*, 2004). Polymorphonuclear neutrophils and macrophages are a main source of exogenous ROS in that they release large quantities of ROS in response to a variety of stimuli. This exogenously produced ROS is crucial in the innate immune system of the host for killing invading bacteria but may also be responsible for tissue injury, when expressed excessively or inappropriately (Lewis and Pollard, 2006). Inflammatory cells are prominent in the stromal compartment of virtually all types of malignancy. These highly versatile cells respond to the presence of stimuli in different parts of tumors (Balkwill and Mantovani, 2001). In an *in vitro* study of rat cells, both macrophages and neutrophils were found to be mutagenic in response to alpha-quartz dust, talc and diesel soot; however, neutrophils appeared to have a greater mutagenic effect (Driscoll *et al.*, 1997). This study found that talc not only increased the ROS generation in the ovarian cells (Fig. 3), but also increased the expression of ROS by the neutrophils (Fig. 4).

Talc has been shown to be ubiquitous in our modern environment (Bremmell and Addai-Mensah, 2005) despite concerns raised about its safety (Janssen, 2004), its role as a possible carcinogen (Cramer *et al.*, 1999; Wong *et al.*, 1999), and its known ability to cause irritation and inflammation (Holthouse and Chleboun, 2001). The data show that talc is capable of increasing cell proliferation, inducing neoplastic transformation of both the normal stromal and epithelial ovarian cells *in vitro*; and increasing ROS generation in these cells as well as the PMN cells.

Cancer chemoprevention is regarded as an efficient strategy to prevent cancer. The most useful cancer chemopreventive compounds will have minimal long-term toxicity, while significantly reducing tumor incidence, delaying tumor onset or preventing tumor progression (Kapadia *et al.*, 2003). It was hypothesized that Pyc, shown to induce apoptosis in various malignant cells (Huang *et al.*, 2005; Huynh and Teel, 2000), could prevent talc-induced neoplastic transformation of normal ovarian cells. It was recently shown that Pyc selectively induced cell death in established malignant ovarian germ cells *in vitro* (Buz'Zard and Lau, 2004). The present study showed that Pyc was capable of inhibiting the above mentioned talc-induced changes. Pretreatment with Pyc prevented the characteristic talc-induced increase in cell viability of GC1a cells (Fig. 5B). Pretreatment with Pyc was able to decrease the ROS generation compared with the respective controls both in a dose- and time-dependent manner (Fig. 7). The data show that pretreatment with Pyc reduced the number of talc-induced transformed colonies in both cell lines (Fig. 6). In the GC1a cells, the decrease in the number of transformed colonies was statistically significant at all concentrations of Pyc (Fig. 6B).

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In conclusion, our *in vitro* data suggest that: (1) talc may contribute to ovarian carcinogenesis in humans by way of inducing aberrant ROS generation and (2) Pyc reduces talc-induced neoplastic transformation of ovarian cells. Taken together, Pyc may prove to be a chemopreventative agent against ovarian carcinogenesis.

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The primary role of iron-mediated lipid peroxidation in the differential cytotoxicity caused by two varieties of talc nanoparticles on A_{549} cells and lipid peroxidation inhibitory effect exerted by ascorbic acid

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ABSTRACT

Talc particles, the basic ingredient in different kinds of talc based cosmetic and pharmaceutical products, pose a health risk to pulmonary and ovarian systems due to domestic and occupational exposures. Two types of talc nanoparticles depending on the source of geographical origin indigenous and commercial talc nanoparticles were assessed for their potential *in vitro* toxicity on A_{549} cells; along with indigenous conventionally used microtalc particles. Cell viability, determined through live/dead staining and 3 (4,5 dimethyl thiazol 2 yl) 2,5 diphenyl tetrazolium bromide (MTT) assay, decreased as a function of concen tration, origin and size of particles. Both varieties of talc nanoparticles differentially induced lipid perox idation (LPO), which was correlated with the pattern of lactate dehydrogenase (LDH) leakage, reactive oxygen species (ROS) generation, and glutathione (GSH) depletion. Relatively higher cytotoxicity of indig enous nanotalc could be attributed to its higher content of iron as compared to commercial nanotalc. The known scavenger of ROS, L ascorbic acid significantly inhibited LPO induction due to talc particles. Data suggest that nanotalc toxicity on A_{549} cells was mediated through oxidative stress, wherein role of iron mediated LPO was much pronounced in differential cytotoxicity.

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1. Introduction

Talc is a magnesium silicate mineral with chemical formula written as $3MgO\ 4SiO_2H_2O$ which corresponds to 4.8% H_2O, 31.7% MgO, and 63.5% SiO_2. It is chemically inert to acids and alkalis and can withstand temperatures up to 1300 °C. In pulverized form it is whiter in appearance. Talc is valued for its extreme softness, smoothness, high lubricating and hiding power and ability to ab sorb oil and grease. Talc is, therefore, used by organized sector of industries because of its valuable properties. Pulverized talc has wide industrial applications in cosmetics as body and face powder; filler in rubber, textile, plastic, asbestos products, polishes and soaps; as a loading agent for paper of all kinds; used in pharmaceu ticals as a carrier of insecticidal and pesticidal dusts.

Since, talc products are marketed in a multitude of grades which have physical or functional characteristics especially suited for particular applications and products, so occupational and con sumer exposures to talc are complex. Talc miners have shown higher rates of lung cancer and other respiratory illnesses from exposure to industrial grade talc, which contains dangerous silica and asbestos (Hollinger, 1990; National Toxicology Program, 1993). The common household hazard posed by talc is inhalation of baby powder by infants (Hollinger, 1990). Talc particles have been found to be translocated after intrapleural administration in rats (Werebe and Pazetti, 1999). Talc particles are able to move through the human reproductive system and become imbedded in the lining of the ovary. Researchers have found talc particles in ovarian tumors and have found that women with ovarian cancer have used talcum powder in their genital area more frequently than healthy women (Henderson et al., 1971; Harlow et al., 1992; Harlow and Hartge, 1995). Numerous studies have shown a strong link between frequent use of talc in the female genital area and ovarian cancer (Heller et al., 1996; Chang and Risch, 1997; Cook et al., 1997; Cramer et al., 1999; Mills et al., 2004; Wild, 2006). In an epidemiologic study aimed to analyze the interactions between talc use and genes involved in detoxification pathway, (viz; glutathione S transferase M1 (GSTM1), glutathione S trans ferase T1 (GSTT1), and N acetyltransferase 2 (NAT2), suggest that women with certain genetic variants may have a higher risk of

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ovarian cancer associated with genital use of talc (Gates et al., 2008).

Nanopowder of talc is a recent introduction and is used for improving quality of many industrial products. Nanopowder of talc is being used in plastics for higher strength and stiffness, better thermal and creep resistance; in papers for higher opacity, better gloss and printing quality; in cosmetics and paints for better gloss, smoother surface, resistance to water and cracking, etc. Owing to their unique nano size, nanoparticles are provided with many special physicochemical properties, and thereby may yield extraordinary hazards for human health (Donaldson et al., 2002; Kipen and Laskin, 2005; Holsapple et al., 2005; Nel et al., 2006; Borm et al., 2006). Since, talc with a multitude of physical and functional characteristics is used for particular applications, so occupational and consumer exposures to talc are likely to vary accordingly. Risk of occupational and environmental exposure to nanoparticles of talc has obviously increased.

Since, physical and functional characteristics of talc and other minerals depend, in part, from one geographical region/source to other, therefore, the first objective of the present study was to evaluate cytotoxicity of talc nanoparticles from the two sources indigenous nanotalc (Indian origin) and commercial nanotalc (American origin) using human bronchoalveolar carcinoma derived cells (A_{549}). Indigenous micro scale talc particle was used for comparative size dependent toxicity with the two types of nanotalc. The second objective was to study the mechanism of cytotoxicity induced by talc nano and micro particles. In the present study, different types of talc particles were dispersed in the cell culture medium at varying concentrations and then exposed to cells. Cytotoxicity was measured by determining cell viability using MTT assay and live dead staining method. To elucidate the possible mechanisms of cytotoxicity, biomarkers for cytotoxicity and oxidative stress, namely lactate dehydrogenase (LDH) leakage in cell culture medium, reactive oxygen species (ROS) generation, intracellular reduced glutathione (GSH) level, and malondialdehyde (MDA) as an indicator of lipid peroxidation and membrane damage, were measured. Antioxidant, ascorbic acid, was used to delineate further the potential mechanism of oxidative stress and as a potential preventive measure. In the toxicity of minerals, the iron content has been a key factor, acting through Fenton reaction and the Haber Weiss cycle. Some metals like Fe, Pb, and Cr was measured in the talc from two sources. A role of differential amount of iron present in indigenous and commercial talc, in the perspective of cytotoxicity and oxidative stress has, therefore, also been established.

2. Materials and methods

2.1. Nanoparticles

Indigenous cosmetic grade talc was collected from Udaipur, Rajasthan, India and prepared into micro and nanoparticles. As a standard reference, Nanopowder of talc (i.e. commercial nanotalc) was purchased from (M.K. Impex Canada, Catalpa Road, Mississauga, Canada). As per the information provided by the supplier, the powder size was 70 120 nm and the country of origin was USA. For indigenous nanotalc a stone of talc was crushed into fine particles and fed into a ball mill (PM 100, Retsch, Germany) and grinded for 5 days at an alternative cycles of grinding (10 min) and halt (30 min) at 350 rpm using a mixture of different sizes of balls. The sizes of nanoparticles were measured by transmission electron microscopy (TEM) and found to be 80 130 nm. Indian talc particles (i.e. indigenous micro talc) 50 65 μm served as negative control for a comparative study on nanotoxicity of indigenous and commercial varieties of nanotalc.

2.2. Chemicals

Fetal bovine serum, Penicillin streptomycin, DMEM F 12 medium, HBSS was purchased from Invitrogen Co. (Carlsbad, CA, USA). MTT [3 (4,5 dimethylthiazol 2 yl) 2,5 diphenyltetrazolium bromide], NADH, Pyruvic acid, L ascorbic acid, glutathione reduced (GSH), o phthalaldehyde (OPT), 2′,7′ dichlorofluorescin diacetate (DCFH DA), 1,1,3,3 tetraethoxypropane (TEP), 2 thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS), Na_2HPO_4, NaH_2PO_4, were obtained from Sigma Aldrich. Ultrapure DI water was prepared using a Milli Q system (Millipore, Bedford, MA, USA). All other chemicals used were of reagent grade.

2.3. Estimation of heavy metals in indigenous and commercial talc

Talc samples were digested in digesting mixture (HNO_3 and perchloric acid in a ratio of 4:1) for 24 h on hot plate in a fume hood. The digested samples were dissolved in 1% HNO_3 and filtered. The filtrate was used for metal analysis by atomic absorption spectroscopy (AAS). Before analysis, AAS was calibrated every time by running at least three standard concentration (1, 3 and 5 mg/L) of each metal. Values have been expressed as% metal content in talc samples.

2.4. Measurement of hydrodynamic size of nanotalc

These particles were suspended in complete cell culture media, ultrasonicated at 30 W for 2 min (Sonics Vibra Cell, India) and a dynamic light scattering (DLS Malvern Instruments USA) performed for particle size distribution in culture media.

2.5. Cell culture and treatment with talc particles

The A_{549} cell line has been established in permanent culture from a human lung adenocarcinoma (Lieber et al., 1976). *In vitro*, these cells are largely differentiated as alveolar epithelial cells, type II (Croute et al., 1990). The A_{549} cells were obtained from National Centre For Cell Science (NCCS), Pune, India. Cells were maintained in DMEM F 12 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and grown at 37 °C in a humidified, 5% CO_2 incubator. For the determination of GSH, MDA, and LDH levels, A_{549} cells were plated into 75 cm^2 flasks at a density of 2.0×10^6 cells per flask in 12 ml culture medium and allowed to attach for 24 h. Then, the freshly dispersed talc nanoparticles suspensions in cell culture medium were prepared and diluted to appropriate concentrations (50, 100, and 200 μg/ml) and immediately applied to the cells in 15 ml culture medium. Cells not exposed to particles served as controls in each experiment. The selection of the 50 200 μg/ml dosage range of talc nanoparticles was based on a preliminary dose response study (data not shown). A dosage level lower than 50 μg/ml did not result cytotoxicity significantly. The 48 h exposure time was chosen for investigation; the responses at 24 h exposure were not as pronounced as that at 48 h. Therefore, all the data presented here is that of 48 h exposure. Throughout the studies presented in this paper, we utilized a particle dose of 20 μg/cm^2 = 100 μg/ml.

2.6. Cell viability assay

Cytotoxicity was measured by determining cell viability using MTT assay and live dead staining method.

2.6.1. MTT assay

Cell proliferation/viability was assessed by the MTT assay as first described by Mossman (1983) and later modified by Hansen et al. (1989). This assay is based on the ability of viable cells, but

not of dead cells, to reduce soluble, yellow 3 (4,5 dimethyl thiazol 2 yl) 2,5 diphenyl tetrazolium bromide (MTT) into insoluble, blue formazon product. Briefly, around 10,000 A_{549} cells per well were plated in 96 well microtiter plates in a 100 μl of medium. The next day, the medium was changed and the cells were treated with talc nanoparticles at 50 , 100 , and 200 μg/ml for 48 h. After the expo sure time completed, the medium was aspirated off and 100 μl MTT laden media (0.5 mg MTT/ml of media without phenol red and serum, filtered through 0.22 μm filter) added and incubated for 2 h. The reaction was stopped and formazan crystal thus formed was solubilised by mixing an equal volume of stop mix solution containing 20% SDS in 50% *N,N* dimethylformamide and left overnight on a shaker. To minimize the interference in absor bance caused by previously dosed talc particles (at concentrations like 50 200 μg/ml obviously resulting in turbidity!), the plates were centrifuged at 3000 rpm for 5 min to settle down the particles and a clear 100 μl supernatant was transferred to other fresh wells of microtiter plate and then absorbance at 570 nm was taken by a microplate reader (Omega Fluostar). Following noncellular back ground (blank consisting of yellow MTT and stop mix solutions) subtraction, all data were normalized to the MTT conversion activ ity of media treated control cells. This value corresponds to 0% de crease in MTT conversion activity and represents 100% cell viability.

2.6.2. Live dead staining (trypan blue exclusion) assay

In addition to the MTT assay, the cell viability was also deter mined by the trypan blue exclusion method. The percentage of non stained live cells was evaluated using a haemocytometer. A total of 200 cells were counted for each measurement.

2.7. LDH leakage

The activity of cytoplasmic LDH released into the culture media was determined with the method described elsewhere (Wroblew ski and LaDue, 1955; Welder et al., 1991). A 100 μl sample from the centrifuged culture media was collected after the cells were treated for 48 h. The LDH activity was assayed in 3.0 ml of reaction mixture with 100 μl of pyruvic acid (2.5 mg/ml phosphate buffer) and 100 μl of NADH (2.5 mg/ml phosphate buffer) and the rest of the volume adjusted with phosphate buffer (0.1 M, pH 7.4). The rate of NADH oxidation was determined by following the decrease in absorbance at 340 nm for 3 min at 30 s interval at 25 °C using a spectrophotometer (Thermo Spectronic). The amount of LDH re leased is represented as LDH activity (IU/L) in culture media.

2.8. Intracellular ROS measurement

The generation of intracellular ROS was measured using 2′,7′ dichlorofluorescin diacetate (DCFH DA) probe (Wang and Joseph, 1999). DCFH DA passively enters the cell where it is broken down into cell impermeable, non fluorescent reduced dichlorofluorescin (DCFH) and diacetate by cellular esterases. Now DCFH becomes oxidized with intracellular ROS to form the highly fluorescent com pound dichlorofluorescin (DCF) that may be cell permeable. Briefly, 10 mM DCFH DA stock solution made in dimethyl sulfoxide (DMSO) was diluted in culture medium without serum or other additive to yield a 100 μM working solution. After 48 h of exposure to talc nanoparticles, the cells in the 12 well plate were washed twice with HBSS and then incubated in 1 ml working solution of DCFH DA at 37 °C for 30 min. The cells were lysed in alkaline solu tion and centrifuged at 3000 rpm. A 200 μl supernatant was trans ferred to black 96 well plate and fluorescence was then read at 480 nm excitation and 520 nm emission using a microplate reader (Omega Fluostar). The intensity of untreated control well was as sumed to be 100% and data is represented in percent of control.

2.9. Determination of intracellular GSH

The cellular content of GSH was quantified by the fluorometric assay of Hissin and Hilf (1976). After exposure, cells were lysed in 20 mM Tris (pH 7.0) by repeated cycles of freeze thaw and centri fuged at 10,000 rpm for 10 min at 4 °C. The supernatant was trans ferred to another tube and protein content was measured. For the determination of intracellular GSH, protein in this supernatant was precipitated at 1% perchloric acid and again centrifuged at 10,000 rpm for 5 min at 4 °C. Now 20 μl sample was mixed with 160 μl of 0.1 M phosphate 5 mM EDTA buffer, pH 8.3 and 20 μl *o* phthalaldehyde (OPT, 1 mg/ml in methanol) in a black 96 well plate. After 2 h incubation at room temperature in the dark, fluo rescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 355 nm, along with similarly pre pared standards of GSH in 1% perchloric acid. Results are expressed as nmol GSH/mg of cellular protein.

2.10. Determination of thiobarbituric acid reactive substances (TBARS)

LPO was assessed by the TBARS assay, which detects mainly malondialdehyde (MDA), an end product of the peroxidation of polyunsaturated fatty acids and related esters. TBARS was mea sured by slight modification of the method of Ohkawa et al. (1979). Subconfluent cells were scraped in 75 cm^2 flasks, washed two times by isotonic trace element free Tris HCl buffer (400 mM, pH 7.3). A 200 μl aliquot of cell suspension was subse quently mixed with 800 μl of LPO assay cocktail containing (0.4% (w/v) thiobarbituric acid, 0.5% (w/v) SDS, 5% (v/v) acetic acid, pH 3.5 and incubated for 60 min at 95 °C. The sample was cooled using tap water and centrifuged at 5000 rpm for 5 min. The absorbance of the supernatants was read at 532 nm against a standard curve prepared using the MDA standard (10 mM 1,1,3,3 tetramethoxy propane in 20 mM Tris HCl, pH 7.4). Results were calculated as nmol of MDA/mg of cellular protein.

2.11. Addition of L ascorbic acid

To test the potential antioxidant effects afforded by ascorbic acid, 1.5 mM was applied to cell culture 30 min before exposure with particles. A dosage of 200 μg/ml of the two varieties of talc was then exposed for 48 h and MDA level was measured as illus trated above.

2.12. Estimation of protein

The total protein concentration was measured by the Bradford method (Bradford, 1976) using a ready to use Bradford reagent (Sigma Aldrich, USA) and bovine serum albumin as the standard.

2.13. Statistics

Data were expressed as the mean ± SD from three independent experiments. One way ANOVA and Dunnett's Multiple Comparison Test was applied using Graph Pad prism (Version 5.0) software for significance testing, using a p value $\leqslant 0.05$.

3. Results

3.1. Iron contamination in talc samples

Indigenous and commercial talc samples were analyzed for con tamination of heavy metals (Fe, Pb, and Cr). The results are given in Table 1. Indigenous talc contained almost 2.3 times higher iron level in comparison to commercial talc. Pb was not in detectable

Table 1
Metal contamination in talc samples.

Name of metal	% Metal content	
	Indigenous talc	Commercial talc
Fe	0.19	0.08
Cr	Not detectable	0.0046
Pd	Not detectable	Not detectable

limit in both the samples. However Cr was present in trace amount in commercial nanotalc.

3.2. Hydrodynamic size of talc nanoparticles in culture media

The size measured by a dynamic light scattering method was the particles hydrodynamic size, which indicates the extent of aggregation of particles in suspension. The measurements have been given in Table 2. Results show that aggregation occurred and the aggregation was not uniform.

3.3. The concentration , size , and origin dependent cytotoxicity of talc particles

The A_{549} cells were exposed with indigenous microtalc (50 65 μm) particles, indigenous talc nanoparticles (80 130 nm) and commercial talc nanoparticles (70 120 nm) for 48 h exposure, and the cell viability was assessed by MTT assay. Cell viability de creased as a function of concentration, size and geographical origin of particles. Cell viability decreased to 93.0%, 91.6%, and 83.6% for indigenous microtalc and 81.6%, 67.0%, and 47.30% for indigenous nanotalc and 88.3%, 77.6%, and 64.0% for commercial nanotalc par ticles when exposed at 50 , 100 , and 200 μg/ml, respectively (Fig. 1). Fig. 2 shows the results on cell viability obtained by trypan blue exclusion test for similar experiment. Cell viability decreased to about 93.0%, 90.6%, and 83.6% for indigenous microtalc and 83.6%, 73.6%, and 57.30% for indigenous nanotalc and 88.6%, 78.6%, and 69.6% for commercial nanotalc particles exposed at 50 , 100 , and 200 μg/ml, respectively. The IC_{50}s evaluated by MTT and trypan blue assay is given in Table 3.

3.4. Cell membrane damage

LDH release, a marker of cell membrane damage, was measured at 50, 100, and 200 μg/ml for the 48 h exposure (Fig. 3). Following exposure to talc particles at concentrations mentioned above, the LDH activity in the culture media is increased in a concentration dependent manner and found to 18.1%, 32.9%, and 61.3%, respec tively for indigenous microtalc and 99.2%, 193.6%, and 275.6%, respectively for indigenous nanotalc and 46.2%, 103.7%, and 178.7%, respectively for commercial nanotalc. The indigenous nan otalc induced a significantly higher ($p < 0.05$) cell membrane dam age when compared with its micro scale size and commercial nanotalc for a particular concentration. For instance, 50 , 100 , and 200 μg/ml exposure of indigenous nanotalc induced 1.4 , 1.44 , and 1.3 fold higher membrane damage when compared with the same concentrations of commercial nanotalc induced mem brane damage. Similarly indigenous nanotalc induced membrane damage was 1.6 , 2.2 , and 2.3 times higher than that of indigenous microtalc.

Table 2
Actual and hydrodynamic sizes of Indigenous and Commercial nanotalc in culture media.

Type of nanoparticles	Actual size (nm)	Hydrodynamic size (nm)
Commercial nanotalc	70–120	800 ± 100
Indigenous nanotalc	80–130	750 ± 120

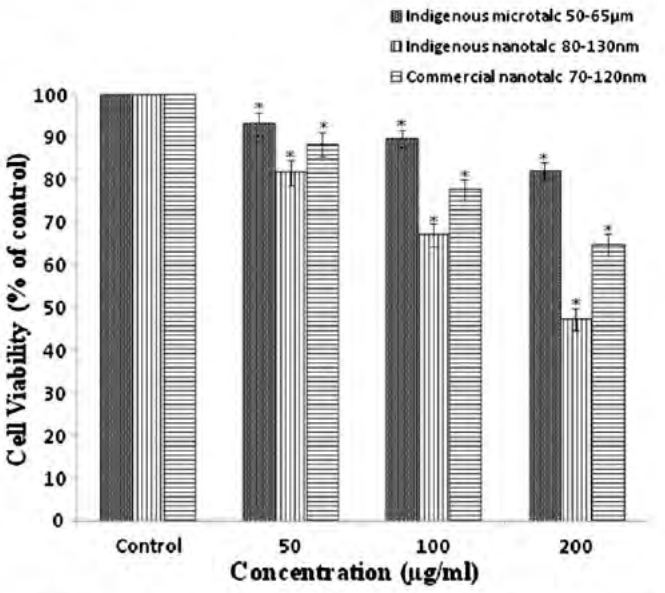

Fig. 1. Viability of A_{549} cells after 48-h exposure to indigenous microtalc, indigenous nanotalc and commercial nanotalc particles evaluated by MTT assay at indicated concentrations. Values are mean ± SD from three independent experiments. Triplicates of each treatment group were used in each independent experiment. *Denotes a significant difference from the control ($p < 0.05$).

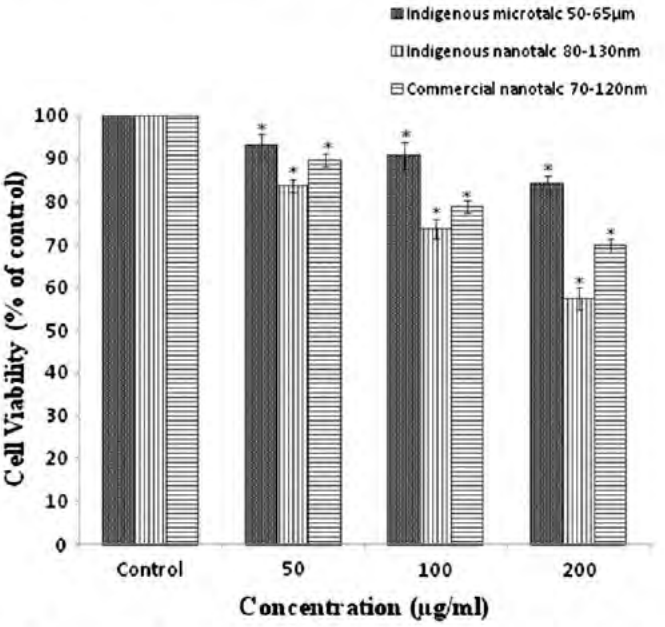

Fig. 2. Viability of A_{549} cells after 48-h exposure to indigenous microtalc, indigenous nanotalc and commercial nanotalc particles evaluated by trypan blue assay at indicated concentrations. Values are mean ± SD from three independent experiments. Triplicates of each treatment group were used in each independent experiment. *Denotes a significant difference from the control ($p < 0.05$).

3.5. ROS generation

The ability of talc micro and nanoparticles to induce intracellu lar oxidant production in A_{549} cells was assessed by measuring DCF fluorescence as a reporter of ROS generation. DCF fluorescence intensity significantly ($p < 0.05$) increased after 48 h exposure to all examined micro and nanoparticles at concentrations of 50 ,

Table 3
IC_{50} values of different talc particles measured by MTT and trypan blue.

Types of talc nanoparticles	IC_{50} by MTT assay (μg/ml)	IC_{50} by trypan blue assay (μg/ml)
Indigenous microtalc (50–65 μm)	600	630
Indigenous nanotalc (80–130 nm)	190	255
Commercial nanotalc (70–120 nm)	277.5	325

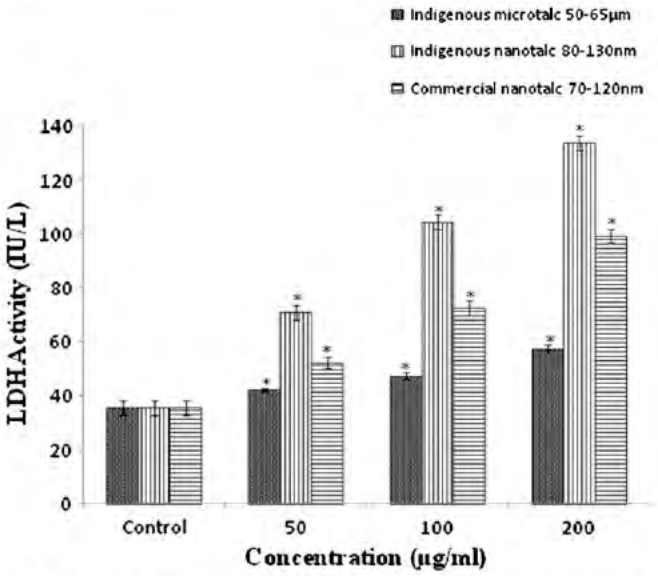

Fig. 3. The LDH activities in the cell culture medium after 48-h exposure to indigenous microtalc, indigenous nanotalc and commercial nanotalc particles at indicated concentrations. Values are mean ± SD from three independent experiments. *Denotes a significant difference from the control ($p < 0.05$).

100 , and 200 μg/ml, and evaluated to be 136%, 155%, and 175%, respectively for indigenous microtalc and 150%, 203%, and 265%, respectively for indigenous nanotalc and 136%, 175%, and 205%, respectively for commercial nanotalc (Fig. 4). The highest fluores cence obtained was that for indigenous nanotalc at 200 μg/ml.

3.6. Cellular GSH level and LPO induced by talc nanoparticles

Following exposure to talc particles at concentrations 50, 100, and 200 μg/ml for 48 h, the intracellular GSH level exhibited a con centration dependent decrease (Fig. 5). The GSH levels were re duced by 3%, 11.56%, and 18.8% for indigenous microtalc and 14.2%, 18.8%, and 25.4% for indigenous nanotalc and 6.6%, 11.5%, and 20.8%, respectively for commercial nanotalc.

In order to elucidate the lipid peroxidation induced by talc par ticles, the MDA concentration was measured. Each type of nano particles elevated the intracellular MDA concentration which was dependent on dosage and source of talc particle origins (Fig. 6). The MDA levels were elevated by 1.3 fold, 1.4 fold, and 1.9 fold, respectively for indigenous microtalc, and 1.6 fold, 2.3 fold, and 3.1 fold, respectively for indigenous nanotalc and 1.4 fold, 1.7 fold, and 2.1 fold, respectively for commercial nanotalc, compared to the control untreated groups.

3.7. Inhibitory effect afforded by ascorbic acid on LPO induced by talc nanoparticles

In an additional set of studies, L ascorbic acid was added to the cells during exposure to micro and nanotalc, each group ex

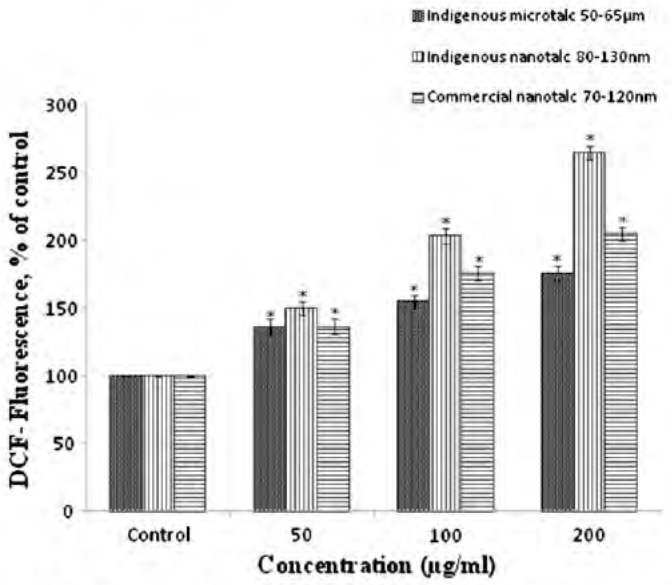

Fig. 4. DCF-fluorescence intensity after 48-h exposure to indigenous microtalc, indigenous nanotalc and commercial nanotalc particles at indicated concentrations. Values are mean ± SD from three independent experiments. *Denotes a significant difference from the control ($p < 0.05$).

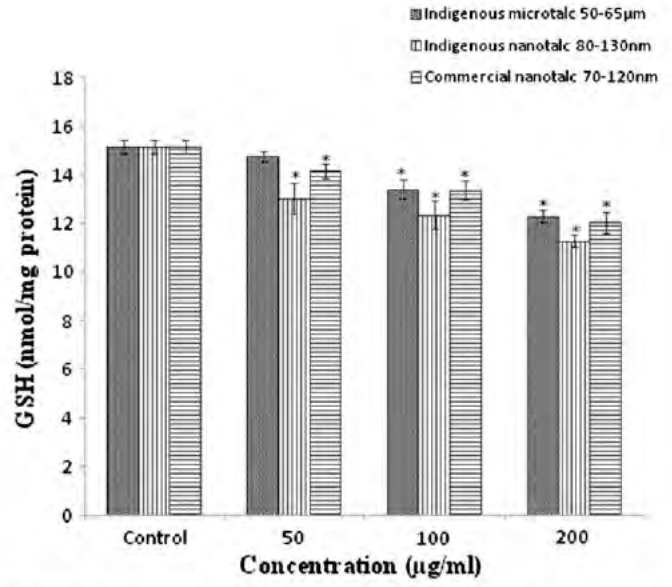

Fig. 5. Cellular GSH levels of A_{549} cells after 48-h exposure to indigenous microtalc, indigenous nanotalc and commercial nanotalc particles at indicated concentrations. Values are mean ± SD from three independent experiments. *Denotes a significant difference from the control ($p < 0.05$).

posed at 200 μg/ml, as a test to determine if the oxidative damage to A_{549} cells could be prevented. Results show that L ascorbic acid effectively prevented the generation of MDA level induced by talc particles (Fig. 7). MDA level was reduced up to control level for indigenous microtalc in the presence of ascorbic acid. When indigenous nanotalc induced MDA was 3.1 fold, in the presence of ascorbic acid it was reduced and found to be 2.1 fold of control. When commercial nanotalc induced MDA was 2.1 fold, in the presence of ascorbic acid it was 1.3 fold of control.

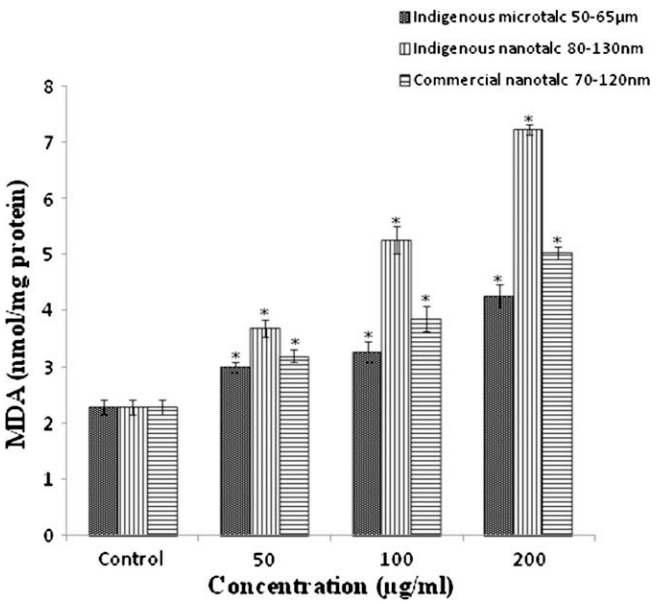

Fig. 6. Cellular MDA levels of A_{549} cells after 48-h exposure to indigenous microtalc, indigenous nanotalc and commercial nanotalc particles at indicated concentrations. Values are mean ± SD from three independent experiments. *Denotes a significant difference from the control ($p < 0.05$).

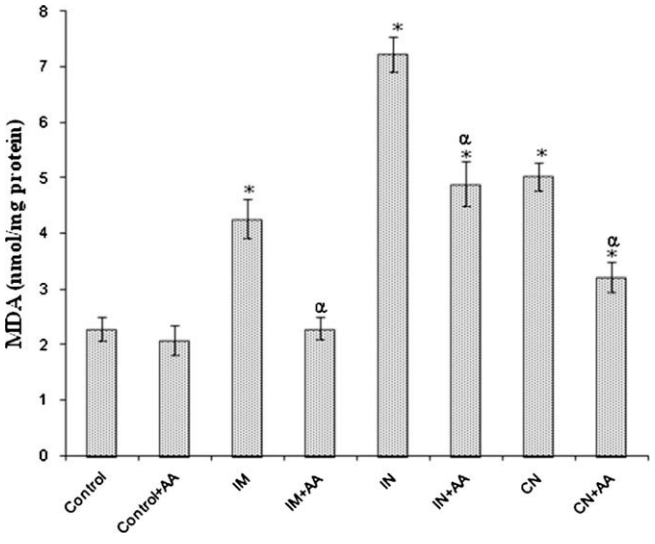

Fig. 7. Showing the inhibitory effect of ascorbic acid on cellular MDA levels of A_{549} cells under indicated conditions of 48-h exposure. #AA (1.5 mM L-ascorbic acid); IM (200 µg/ml indigenous microtalc); IN (200 µg/ml indigenous nanotalc); CN (200 µg/ml commercial nanotalc) Values are mean ± SD from three independent experiments. *Denotes a significant difference from the control ($p < 0.05$). α indicates the significant inhibitory effect of ascorbic acid (AA) on lipid peroxidation versus either, IM, IN or CN.

4. Discussion

At present, an *in vitro* toxicological study of talc nanoparticles is lacking. In this study, the cytotoxicity of two types of talc nanoparticles was investigated in cultured human bronchoalveolar carcinoma derived cells (A_{549}). This cell line has been widely used in *in vitro* cytotoxicity studies (Huang et al., 2004; Bakand et al., 2006). Present study showed that the two types of talc nanoparticles caused significant reduction in cell viability as a function of concentration and their iron content. The talc nanoparticles from two sources induced the enhanced generation of ROS and MDA production. Consequently, redundant free radicals would interact with biomolecules including proteins, enzymes, membrane lipids and even DNA which could be oxidized, modified, destructured and ultimately dysfunctioned (Marnett, 2000; Hensley and Floyd, 2002).

Oxidative stress has been suggested to play an important role in the mechanism of toxicity of a number of compounds whether by production of free radicals or by depleting cellular antioxidant capacity. Cellular integrity is affected by oxidative stress when the production of ROS overwhelms antioxidant defense mechanism (Halliwell et al., 1992; Chen and Yu, 1994). ROS are oxygen containing molecules, such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot}$), and hydroxyl radical ($HO^{\cdot}$), that have a greater chemical activity than molecular oxygen. ROS are generated in many inflammatory conditions in the lung and have been associated with cell injury and apoptosis (Anderson et al., 1994; Meyer et al., 1993). Many other studies have shown that nanoparticles may produce toxicity by generating ROS. Recently, Buz'Zard and Lau (2007) have reported enhanced ROS generation in human ovarian cell culture and have found an increased cell proliferation and neoplastic transformation of human ovarian stromal and epithelial cells exposed with talc. In the present study too, talc micro and nanoparticles induced significantly higher ROS generation compared with untreated A_{549} cells when using the fluorescent dichlorofluorescin probe. Moreover, indigenous nanotalc resulted higher ROS generation than commercial nanotalc.

GSH is the most abundant nonproteinous tripeptide containing a sulfhydryl group in virtually all cells, and it plays a significant role in many biological processes. It also constitutes the first line of the cellular defense mechanism against oxidative injury and is the major intracellular redox buffer in ubiquitous cell types (Meister, 1989). GSH acts as a cosubstrate in the GSH peroxidase catalyzed reduction of hydrogen peroxide or lipid peroxides (Forman et al., 1997) leading to its depletion. Previous studies demonstrated that ROS generation following GSH depletion caused mitochondrial damage (Martensson et al., 1989; Meister, 1995), which has been implicated in apoptosis (Green and Reed, 1998). There was a significant depletion of GSH between the control and the treated groups except for indigenous microtalc at 50 µg/ml. In terms of GSH depletion, indigenous nanotalc was found to be the most toxic.

In the toxicity mechanism of minerals, the iron content has been a key factor. Iron dependent ROS generation from fibers results in the generation of hydroxyl radicals through the Fenton reaction and the Haber Weiss cycle. Iron dependent LPO could be important, since this process requires redox cycling of iron and does not necessarily require H_2O_2 or ROS (Halliwell and Gutteridge, 1990). Indeed, iron has a key role in both the initiation and propagation of LPO, leading to the generation of peroxyl and alkoxyl radicals as well as lipid peroxides (Halliwell and Gutteridge, 1990). It has been known for several years that the surface iron (II) or leachable iron (II) on mineral surfaces reduces molecular oxygen to superoxide anion, which then dismutates to hydrogen peroxide. In the presence of asbestos or silica, hydrogen peroxide and superoxide react via a Fenton like reaction driven by iron to form the potent hydroxyl radical *in vitro* leading to cellular LPO (Mossman et al., 1996). Since, LPO is a sensitive parameter for toxic effects of various environmental pollutants with oxidative properties (Krug and Culig, 1991; Beck Speier et al., 2001; Oberdorster, 2004; Sayes et al., 2005); the authors suspected that the relatively high iron content in both the nanotalc may play a key role to yield higher ROS and in turn caused higher LPO. There are other nanomaterials, such as C_{60}, which mediates cytotoxicity primarily through lipid peroxidation (Sayes et al., 2005; Isakovic et al., 2006) whereas carboxyfullerenes (made by certain surface modifications of C_{60}) have been shown to impart cytoprotective activity by eliminating reactive oxygen species (ROS) and antago

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nizing the effects of the oxidative stress dependent cytotoxicity (Dugan et al., 1997, 2001; Bogdanovic et al., 2004; Isakovic et al., 2006). Recently Scarfì et al. (2009) has reported that plasma membrane contact with quartz, a kind of silica, is sufficient to trigger membrane LPO, TNF α release and cell death in mouse macrophage cell line RAW 264.7. The authors hypothesize that contact of particles with cell membranes initiate ROS generation and LPO in a ratio of amount of iron present in talc nanoparticles.

For a given mass compared with larger particles, the ratio of surface to total atoms or molecules increases exponentially with decreasing particle size. Particle size is thereby an essential determinant of the fraction of reactive groups on particle surface (Oberdorster et al., 2005; Nel et al., 2006). For example, several studies found that ultrafine particles of titania are more toxic than its larger counterparts having the same chemical composition (Donaldson et al., 1998; Gilmour et al., 1997; Oberdorster et al., 1992, 1995; Oberdorster, 1996, 2000). Similarly, surface area dependent induction of oxidative stress and consequently, proinflammatory effects have been found to correlate in case of polystyrene particles by Brown et al. (2001) and Lin et al. (2006) have reported higher toxicity of the two sizes (15 and 46 nm) of silica nanoparticles than micro silica (5 μm) on A_{549} cells. Here two sizes (15 and 46 nm) of silica nanoparticles induced no significant differences in the toxicity and similar was the case in a study done by Sayes et al. (2006), where smaller nanoparticles of titania had effects comparable to larger nanoparticles of titania but showed a phase dependent differential toxicity where anatase titania (photoactive phase), able to generate ROS more strongly, was 100 times more toxic than an equivalent sample of rutile titania. In the present study, both nanoparticles would have been resulted differential surface iron activity per given mass resulting in differential toxicity. When indigenous nanotalc induced toxicity is compared with indigenous microtalc, size dependent factor becomes apparent because all the compositional factors are constant. But when commercial nanotalc (having larger surface area but lower iron content) induced toxicity is compared with indigenous microtalc, the results show a complex function of size and impurities. Since, micro talc size is very large (50 65 μm), than commercial nanotalc (70 120 nm), perhaps size becomes the primary determinant of toxicity, resulting in higher toxicity of commercial nanotalc than indigenous micro talc.

Another pathway of free radical generation by asbestos, silica or particulates like these (e.g. talc particles) occurs via an oxidative burst when fibers and particles are phagocytised by AMs or other cell types, including alveolar epithelial cells and fibroblasts (Churg, 1996). Phagocytic cells can endocytose small particles, whereas bigger crystals and fibers are subject to so called "frustrated phagocytosis". Experimental studies suggest that in *in vivo* conditions "frustrated phagocytosis" appears to have a dramatic influence on the sustained generation of ROS (Hansen and Mossman, 1987; Vallyathan et al., 1992). Repeated "frustrated phagocytosis" would be expected to attract more phagocytes, resulting in chronic enhanced generation of ROS, which in turn contribute to inflammasome activation, resulting in the secretion of IL 1β leading to the initiation of pulmonary fibrosis (Dostert et al., 2008; Cassel et al., 2008). Since, talc and asbestos are physically and chemically similar, found together in nature and being particulate structure like silica and asbestos, talc particles may also generate ROS through activation of NADPH oxidase by frustrated phagocytosis, leading to the initiation of so called talcosis particularly in occupationally exposed workers.

Antioxidants, such as α tocopherol, uric acid and L ascorbic acid, typically prevent cellular damages caused by oxygen radicals by acting as ROS scavengers (Packer et al., 1979; Burton and Ingold, 1981). Ascorbic acid (or vitamin C) acts as a potent water soluble antioxidant in biological fluids (Frei et al., 1989, 1990) by scavenging physiologically relevant ROS and reactive nitrogen species (RNS) (Halliwell, 1996). However, it should be noted that antioxidative potential of ascorbic acid has not been validated in certain conditions (Bowry et al., 1992; Poulsen et al., 1998; Levine et al., 1998). Ascorbic acid contributes significantly to cellular antioxidation as a water soluble chain breaking radical scavenger (Asard, 2008) and to the recycling of plasma membrane α tocopherol (vitamin E) via the reduction of the α tocopheroxyl radical (Aguirre and May, 2008). The latter activity may assist ascorbic acid to protect against LPO in membranes (May et al., 1998). We, therefore, tested the LPO preventive potential of antioxidant L ascorbic acid, on nanotalc and microtalc challenged A_{549} cells. Results show that 1.5 mM L ascorbic acid effectively, but not completely, inhibited MDA level induced by talc nanoparticles. Determining the optimum concentration of ascorbic acid that might completely suppress LPO without causing any side effect is a matter of concern (Halliwell, 1999) and the evaluation of interrelationship between LPO and chelating effect of iron present on the surface of talc particles by deferoxamine mesylate on LPO is under investigation. Oxidative stress is known to elicit varying effects on the activity of antioxidant enzymes. The three primary scavenger enzymes involved in detoxifying ROS in mammalian systems are catalase, superoxide dismutase and glutathione peroxidase (MatÉs et al., 1999). For example the activity of GPx can provide important clue about the consumption rate of GSH in enzymatic detoxification of ROS. The activity of antioxidant enzymes can therefore provide further insight in understanding the mechanism of toxicity caused by talc particles and is currently under investigation.

5. Conclusion

We have presented a preliminary data on the toxicity response elicited by the two types of talc nanoparticles, depending on their different geological origin. Since, talc with a multitude of physical and functional characteristics due to different geological context and deposits, is used for particular applications, so occupational and consumer exposures to talc and its toxic effects are likely to vary accordingly, which is obvious in this study. The cytotoxicity seems to be due to primarily through induction of LPO, as a potential mechanism of toxicity discussed above. Addition of 1.5 mM of L ascorbic acid, a ROS scavenger, significantly, though not completely, reduced LPO. Data clearly suggest that exposure of talc, particularly nanopowder, should be protected in humans at risk of occupational as well as domestic exposure.

Acknowledgments

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Exhibit 96

Case 3:16-md-02738-MAS-RLS Document 9899-1 Filed 05/30/19 Page 54 of 218 PageID: 75125

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Molecular Basis Supporting the Association of Talcum Powder Use With Increased Risk of Ovarian Cancer

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Abstract

Genital use of talcum powder and its associated risk of ovarian cancer is an important controversial topic. Epithelial ovarian cancer (EOC) cells are known to manifest a persistent prooxidant state. Here we demonstrated that talc induces significant changes in key redox enzymes and enhances the prooxidant state in normal and EOC cells. Using real-time reverse transcription polymerase chain reaction and enzyme-linked immunosorbent assay, levels of CA-125, caspase-3, nitrate/nitrite, and selected key redox enzymes, including myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GSR), were determined. TaqMan genotype analysis utilizing the QuantStudio 12K Flex was used to assess single-nucleotide polymorphisms in genes corresponding to target enzymes. Cell proliferation was determined by MTT proliferation assay. In all talc-treated cells, there was a significant dose-dependent increase in prooxidant iNOS, nitrate/nitrite, and MPO with a concomitant decrease in antioxidants CAT, SOD, GSR, and GPX ($P < .05$). Remarkably, talc exposure induced specific point mutations that are known to alter the activity in some of these key enzymes. Talc exposure also resulted in a significant increase in inflammation as determined by increased tumor marker CA-125 ($P < .05$). More importantly, talc exposure significantly induced cell proliferation and decreased apoptosis in cancer cells and to a greater degree in normal cells ($P < .05$). These findings are the first to confirm the cellular effect of talc and provide a molecular mechanism to previous reports linking genital use to increased ovarian cancer risk.

Keywords

talc, epithelial ovarian cancer, oxidative stress, single-nucleotide polymorphism, cell proliferation

Introduction

Ovarian cancer is the most lethal gynecologic malignancy and ranks fifth in cancer deaths among women diagnosed with cancer.[1] Epithelial ovarian cancer (EOC) has long been considered a heterogeneous disease with respect to histopathology, molecular biology, and clinical outcome.[1,2] Although surgical techniques and treatments have advanced over the years, the prognosis of EOC remains poor, with a 5-year survival rate of 50% in advanced stage.[2] This is largely due to the lack of early warning symptoms and screening methods and the development of chemoresistance.[1,2] Moreover, ovarian cancer is known to be associated with germline mutations in the *BRCA1* or *BRCA2* genes, but with a rate of only 20 % to 40%, suggesting the presence of other unknown mutations in other predisposition genes.[3] Additional genetic variations including single-nucleotide polymorphisms (SNPs) have been hypothesized to act as low to moderate penetrant alleles that contribute to ovarian cancer risk.[3,4]

The pathophysiology of EOC is not fully understood but has been strongly associated with inflammation and the resultant oxidative stress.[5] We have previously characterized EOC cells to manifest a persistent prooxidant state as evident by the upregulation of key oxidants and downregulation of key antioxidants, which is further enhanced in chemoresistant EOC cells.[6] The expression of key prooxidant/inflammatory enzymes such as inducible nitric oxide synthase (iNOS), nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, and myeloperoxidase (MPO), as well as an increase in nitric oxide (NO) levels, was increased in EOC tissues and cells.[6] Additionally, we have shown that EOC cells manifest lower apoptosis, which

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was markedly induced by inhibiting iNOS, indicating a strong link between apoptosis and NO/iNOS pathways in these cells.[6]

The cellular redox balance is maintained by key antioxidants including catalase (CAT), superoxide dismutase (SOD), or by glutathione peroxidase (GPX) coupled with glutathione reductase (GSR).[5] Other important scavengers include thioredoxin coupled with thioredoxin reductase, and glutaredoxin, which utilizes glutathione (GSH) as a substrate.[7] We have previously reported that a genotype switch in key antioxidants is a potential mechanism leading to the acquisition of chemoresistance in EOC cells.[7] We have studied the effects of genetic polymorphisms in key redox genes on the acquisition of the oncogenic phenotype in EOC cells, including genes that control the levels of cellular reactive oxygen species and oxidative damage and SNPs for genes involved in carcinogen metabolism (detoxification and/or activation), antioxidants, and DNA repair pathways.[4,6] Several function-altering SNPs have been identified in key antioxidants, including CAT, GPX, GSR, and SOD.[4]

Several studies have suggested the possible association between genital use of talcum powder and risk of EOC.[7-12] Association between the use of cosmetic talc in genital hygiene and ovarian cancer was first described in 1982 by Cramer et al, and many subsequent studies supported this finding.[7-12] Talc and asbestos are both silicate minerals; the carcinogenic effects of asbestos have been extensively studied and documented in the medical literature.[7-12] Asbestos fibers in the lung initiate an inflammatory and scarring process, and it has been proposed that ground talc, as a foreign body, might initiate a similar inflammatory response.[7] The objective of this study was to determine the effects of talcum powder on the expression of key redox enzymes, CA-125 levels, and cell proliferation and apoptosis in normal and EOC cells.

Material and Methods

Cell Lines

Ovarian cancer cells SKOV-3 (ATCC), A2780 (Sigma Aldrich, St Louis, Missouri), and TOV112D (a kind gift from Gen Sheng Wu at Wayne State University, Detroit, Michigan) and normal cells human macrophages (EL-1; ATCC, Manassas, Virginia), human primary normal ovarian epithelial cells (Cell Biologics, Chicago, Illinois), human ovarian epithelial cells (HOSEpiC; ScienCell Research Laboratories, Inc, Carlsbad, California), and immortalized human fallopian tube secretory epithelial cells (FT33; Applied Biological Materials, Richmond, British Columbia, Canada) were used. All cells were grown in media and conditions following manufacturer's protocol. EL-1 cells were grown in IMDM media (ATCC) supplemented with 0.1 mM hypoxanthine and 0.1 mM thymidine solution (H-T, ATCC) and 0.05 mM β-mercaptoethanol. SKOV-3 EOC cells were grown in HyClone McCoy's 5A medium (Fisher Scientific, Waltham, Massachusetts), A2780 EOC cells were grown in HyClone RPMI-1640 (Fisher Scientific), and both TOV112D EOC cells were grown in MCDB105 (Cell Applications, San Diego, California) and Medium 199 (Fisher Scientific; 1:1). All media were supplemented with fetal bovine serum (Innovative Research, Novi, Michigan) and penicillin/streptomycin (Fisher Scientific), per their manufacturer specifications. Human primary normal ovarian epithelial cells were grown in complete human epithelial cell medium (Cell Biologics).

Treatment of Cells

Talcum baby powder (Johnson & Johnson, New Brunswick, NJ, #30027477, Lot#13717RA) was dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich) at a concentration of 500 mg in 10 mL and was filtered with a 0.2 µm syringe filter (Corning). Sterile DMSO was used as a control for all treatments. Cells were seeded in 100-mm cell culture dishes (3×10^6) and were treated 24 hours later with 5, 20, or 100 µg/mL of talc for 72 hours. Cell pellets were collected for RNA, DNA, and protein extraction. Cell culture media were collected for CA-125 analysis by enzyme-linked immunosorbent assay (ELISA).

Real-Time Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from all cells using the RNeasy mini kit (Qiagen, Valencia, California). Measurement of the amount of RNA in each sample was performed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts). A 20 µL complementary DNA reaction volume containing 0.5 µg RNA was prepared using the SuperScript VILO Master Mix Kit (Life Technologies, Carlsbad, California). Optimal oligonucleotide primer pairs were selected for each target using Beacon designer (Premier Biosoft, Inc; Table 1). Quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using the EXPRESS SYBR GreenER qPCR supermix kit (Life Technologies) and the Cepheid 1.2f detection system (Sunnyvale, CA) previously described.[6] Standards with known concentrations and lengths were designed specifically for β-*actin* (79 bp), *CAT* (105 bp), *NOS2* (89 bp), *GSR* (103 bp), *GPX1* (100 bp), *MPO* (79 bp), and *SOD3* (84 bp), allowing for construction of a standard curve using a 10-fold dilution series.[6] All samples were normalized to β-*actin*. A final melting curve analysis was performed to demonstrate specificity of the PCR product.

Protein Detection

Cell pellets were lysed utilizing cell lysis buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptin) containing a cocktail of protease inhibitors. Samples were centrifuged at 13 000 rpm for 10 minutes at 4°C. Total protein concentration of cell lysates from control and talc-treated cells was measured with the Pierce BCA protein assay kit (Thermo Scientific, Rockford, Illinois).

Table 1. Real Time RT PCR Oligionucleotide Primers.

Accession Number	Gene	Sense (5′ 3′)	Antisense (3′ 5′)	Amplicon (bp)	Annealing Time (seconds) and Temperature (°C)
NM 001101	β *actin*	ATGACTTAGTTGCGTTACAC	AATAAAGCCATGCCAATCTC	79	10, 64
NM 001752	*CAT*	GGTTGAACAGATAGCCTTC	CGGTGAGTGTCAGGATAG	105	10, 63
NM 003102	*SOD3*	GTGTTCCTGCCTGCTCCT	TCCGCCGAGTCAGAGTTG	84	60, 64
NM 000637	*GSR*	TCACCAAGTCCCATATAGAAATC	TGTGGCGATCAGGATGTG	116	10, 63
NM 000581	*GPX1*	GGACTACACCCAGATGAAC	GAGCCCTTGCGAGGTGTAG	91	10, 66
NM 000625	*NOS2*	GAGGACCACATCTACCAAGGAGGAG	CCAGGCAGGCGGAATAGG	89	30, 59
NM 000250	*MPO*	CACTTGTATCCTCTGGTTCTTCAT	TCTATATGCTTCTCACGCCTAGTA	79	60, 63

Abbreviation: RT-PCR, reverse transcription polymerase chain reaction.

Detection of Protein/Activity by ELISA

The following ELISA kits were used (Cayman Chemical, Ann Arbor, Michigan): CAT, SOD, GSR, GPX, and MPO. Nitrite (NO_2^-)/nitrate (NO_3^-) were determined spectrophotometrically by Griess assay as previously reported.[6] CA-125 protein levels were measured in cell media by ELISA (Ray Biotech, Norcross, Georgia).

TaqMan SNP Genotyping Assay

DNA was isolated utilizing the EZ1 DNA tissue kit (Qiagen) for EOC cells. The TaqMan SNP genotyping assay set (Applied Biosystems, Carlsbad, California; NCBI dbSNP genome build 37, MAF source 1000 genomes) was used to genotype the SNPs (Table 1). The Applied Genomics Technology Center (AGTC, Wayne State University) performed these assays. Analysis was done utilizing the QuantStudio 12 K Flex real-time PCR system (Applied Biosystems).

Cell Proliferation and Apoptosis

Cell proliferation was assessed with the TACS MTT cell proliferation assay (Trevigen, Gaithersburg, Maryland) after treatment with talc (100 µg/mL) for 24 hours. The Caspase-3 Colorimetric Activity Assay Kit (Chemicon, Temecula, California) was used to determine levels of caspase-3 activity after treatment of normal and EOC cells with various doses of talc as previously described.[6] Equal concentrations of cell lysate were used. The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA. The free pNA can be quantified using a spectrophotometer or a microtiter plate reader at 405 nm. Comparison of the absorbance of pNA from an apoptotic sample with its control allows determination of the percentage increase in caspase-3 activity.

Statistical Analysis

Normality was examined using the Kolmogorov-Smirnov test and by visual inspection of quantile–quantile plots. Because most of the data were not normally distributed, differences in distributions were examined using the Kruskal-Wallis test. Generalized linear models were fit to examine pairwise differences in estimated least squares mean expression values by exposure to 0, 5, 20, or 100 µg/mL of talc. We used the Tukey-Kramer adjustment for multiple comparisons, and the regression models were fit using log2 transformed analyte expression values after adding a numeric constant "1" to meet model assumptions while avoiding negative transformed values. P values below .05 are statistically significant.

Results

Talc Treatment Decreased the Expression of Antioxidant Enzymes SOD and CAT in Normal and EOC Cells

Real-time RT-PCR and ELISA assays were utilized to determine the CAT and SOD messenger RNA (mRNA) and protein levels in cells before and after 72 hours talc treatment, respectively (Figure 1). The CAT (Figure 1A and C) and SOD (Figure 1B and D) mRNA and protein levels were significantly decreased in a dose-dependent manner in talc-treated cells compared to controls ($P < .05$).

Talc Treatment Increased the Expression of Prooxidants iNOS, NO_2^- /NO_3^-, and MPO in Normal and EOC Cells

Real-time RT-PCR and NO_2^- /NO_3^- assays were utilized to determine the iNOS mRNA and NO levels in cells before and after 72 hours talc treatment, respectively (Figure 2). The iNOS mRNA and NO levels were significantly increased in a dose-dependent manner in talc-treated cells as compared to their controls (Figure 2A and C, $P < .05$). As expected, there was no detectable MPO in normal ovarian and fallopian tube cells, and thus, talc treatment did not have any effect. However, MPO mRNA and protein levels were significantly increased in a dose-dependent manner in talc-treated ovarian cancer cells and macrophages compared to controls (Figure 2B and D, $P < .05$).

Talc Treatment Decreased the Expression of Antioxidant Enzymes, GPX and GSR, in Normal and EOC Cells

Real-time RT-PCR and ELISA assays were utilized to determine the GPX and GSR mRNA and protein levels in cells before and

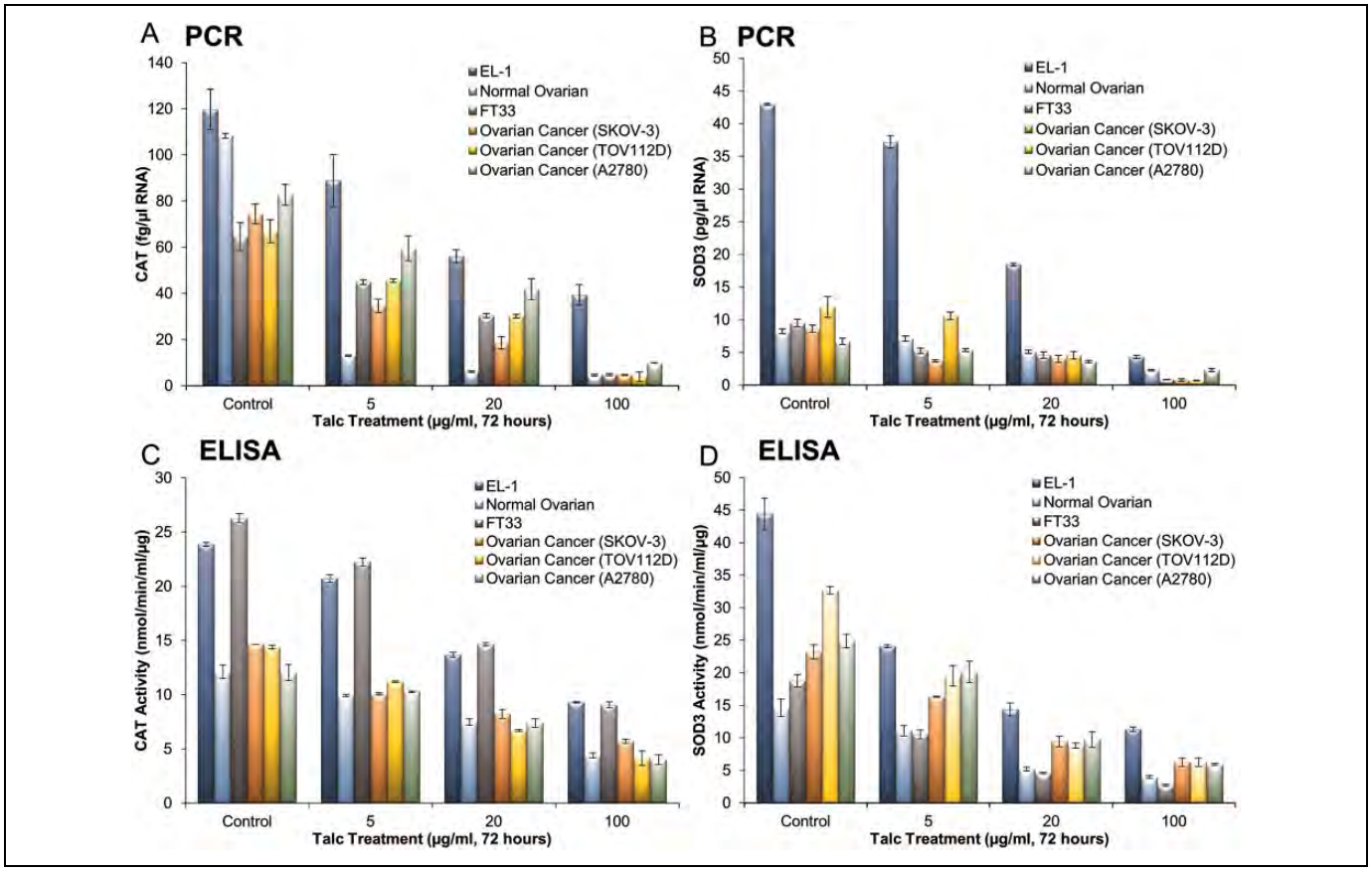

Figure 1. Decreased expression and activity of key antioxidant enzymes, CAT and SOD3. The mRNA (real time RT PCR) and protein/activity levels (ELISA) of CAT (A and C) and SOD3 (B and D) were determined in macrophages (EL 1), human primary ovarian epithelial cells (normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV 3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 72 hours. Experiments were performed in triplicate. Expression is depicted as the mean, with error bars representing standard deviation. All changes in response to talc treatment were significant ($P < .05$) in all cells and in all doses as compared to controls. CAT indicates catalase; SOD3, superoxide dismutase 3; mRNA, messenger RNA; RT PCR, reverse transcription polymerase chain reaction; ELISA, enzyme linked immunosorbent assay.

after 72 hours of talc treatment, respectively (Figure 3). The GPX (Figure 3A and C) and GSR (Figure 3B and D) mRNA and protein levels were significantly decreased in a dose-dependent manner in talc-treated cells compared to controls ($P < .05$).

Talc Exposure Induced Known Genotype Switches in Key Oxidant and Antioxidant Enzymes

Talc treatment was associated with a genotype switch in *NOS2* from the common C/C genotype in untreated cells to T/T, the SNP genotype, in talc-treated cells, except in A2780 and TOV112D (Table 2). Additionally, the observed decrease in CAT expression and activity was associated with a genotype switch from common C/C genotype in CAT in untreated cells to C/T, the SNP genotype, in TOV112D and all normal talc-treated cells. However, there was no detectable genotype switch in CAT in A2780, SKOV3, and TOV112D (Table 2). Remarkably, there was no observed genotype switch in the selected SNP for SOD3 and GSR in all talc-treated cells. All cells, except for HOSEpiC cells, manifest the SNP genotype of *GPX1* (C/T). Intriguingly, talc treatment reversed this SNP genotype to the normal genotype (Table 2).

Talc Treatment Increased CA-125 Levels in Normal and EOC Cells

CA-125 ELISA assay was performed in protein isolated from cell media before and after talc treatment. CA-125 levels were significantly increased in a dose-dependent manner in all cells (Figure 4, $P < .05$). There was no detectable CA-125 protein in macrophages.

Talc Treatment Increased Cell Proliferation and Decreased Apoptosis

MTT cell proliferation assay was used to determine cell viability, and caspase-3 activity assay was utilized to determine apoptosis of all cell lines after 24 hours of talc treatment (Figure 5). Cell proliferation was significantly increased from the baseline in all talc-treated cells ($P < .05$), but to a greater degree in normal

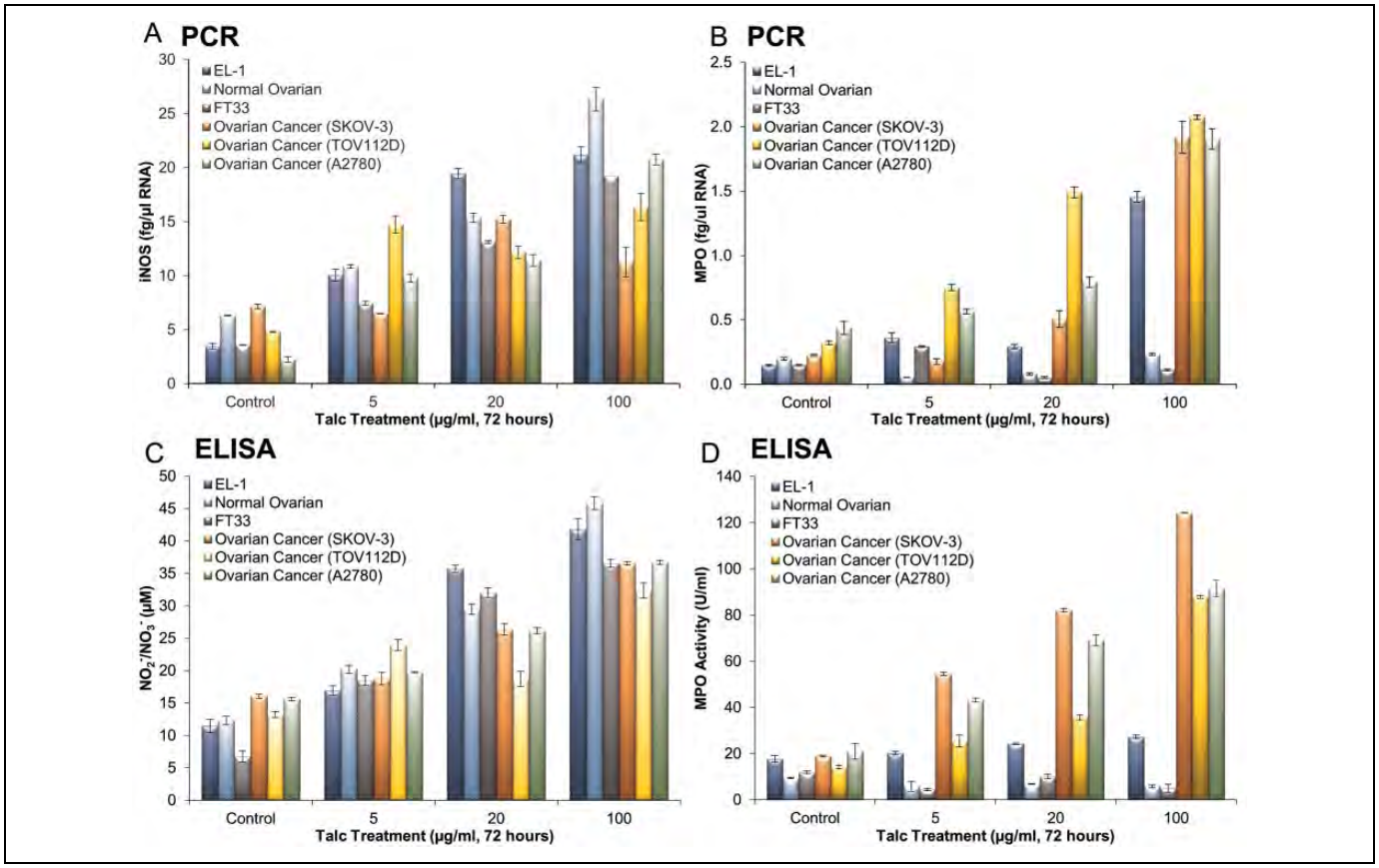

Figure 2. Increased expression and activity of key prooxidants, iNOS, NO_2^-/NO_3^-, and MPO. The mRNA (real time RT PCR) and protein/activity levels (ELISA) of iNOS (A and C) and MPO (B and D) were determined in macrophages (EL 1), human primary ovarian epithelial cells (normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV 3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 72 hours. As expected, there was no detectable MPO in normal ovarian and fallopian tube cells, and thus, talc treatment did not have any effect. Experiments were performed in triplicate. Expression is depicted as the mean, with error bars representing standard deviation. All changes in response to talc treatment were significant ($P < .05$) in iNOS and MPO positive cells and in all doses as compared to controls. iNOS indicates inducible nitric oxide synthase; MPO, myeloperoxidase; mRNA, messenger RNA; RT PCR, reverse transcription polymerase chain reaction; ELISA, enzyme linked immunosorbent assay.

as compared to cancer cells. As anticipated, caspase-3 was significantly reduced in cancer as compared to normal cells. Talc treatment resulted in decreased caspase-3 activity in all cells as compared to controls (Figure 6, $P < .05$), indicating a decrease in apoptosis.

Discussion

The claim that regular use of talcum powder for hygiene purpose is associated with an increased risk of ovarian cancer is based on several reports confirming the presence of talc particles in the ovaries and other parts of the female reproductive tract as well as in lymphatic vessels and tissues of the pelvis.[7-12] The ability of talc particles to migrate through the genital tract to the distal fallopian tube and ovaries is well accepted.[10] To date, the exact mechanism is not fully understood, though several studies have pointed toward the peristaltic pump feature of the uterus and fallopian tubes, which is known to enhance transport of sperm into the oviduct ipsilateral to the ovary bearing the dominant follicle.[8-12]

There are reports supporting the epidemiologic association of talc use and risk of ovarian cancer.[11,12] Recent studies have shown that risks for EOC from genital talc use vary by histologic subtype, menopausal status at diagnosis, hormone therapy use, weight, and smoking. These observations suggest that estrogen and/or prolactin may play a role via macrophage activity and inflammatory response to talc. There has been debate as to the significance of the epidemiologic studies based on the fact that the reported epidemiologic risk of talc use and risk of ovarian cancer, although consistent, are relatively modest (30%-40%), and there is inconsistent increase in risk with duration of use. This observation is due, in part, to the challenges in quantifying exposure as well as the failure of epidemiological studies to obtain necessary information about the frequency and duration of usage.[11-13]

In this study, we have shown beyond doubt that talc alters key redox and inflammatory markers, enhances cell proliferation, and inhibits apoptosis, which are hallmarks of ovarian cancer. More importantly, this effect is also manifested by talc in normal cells, including surface ovarian epithelium,

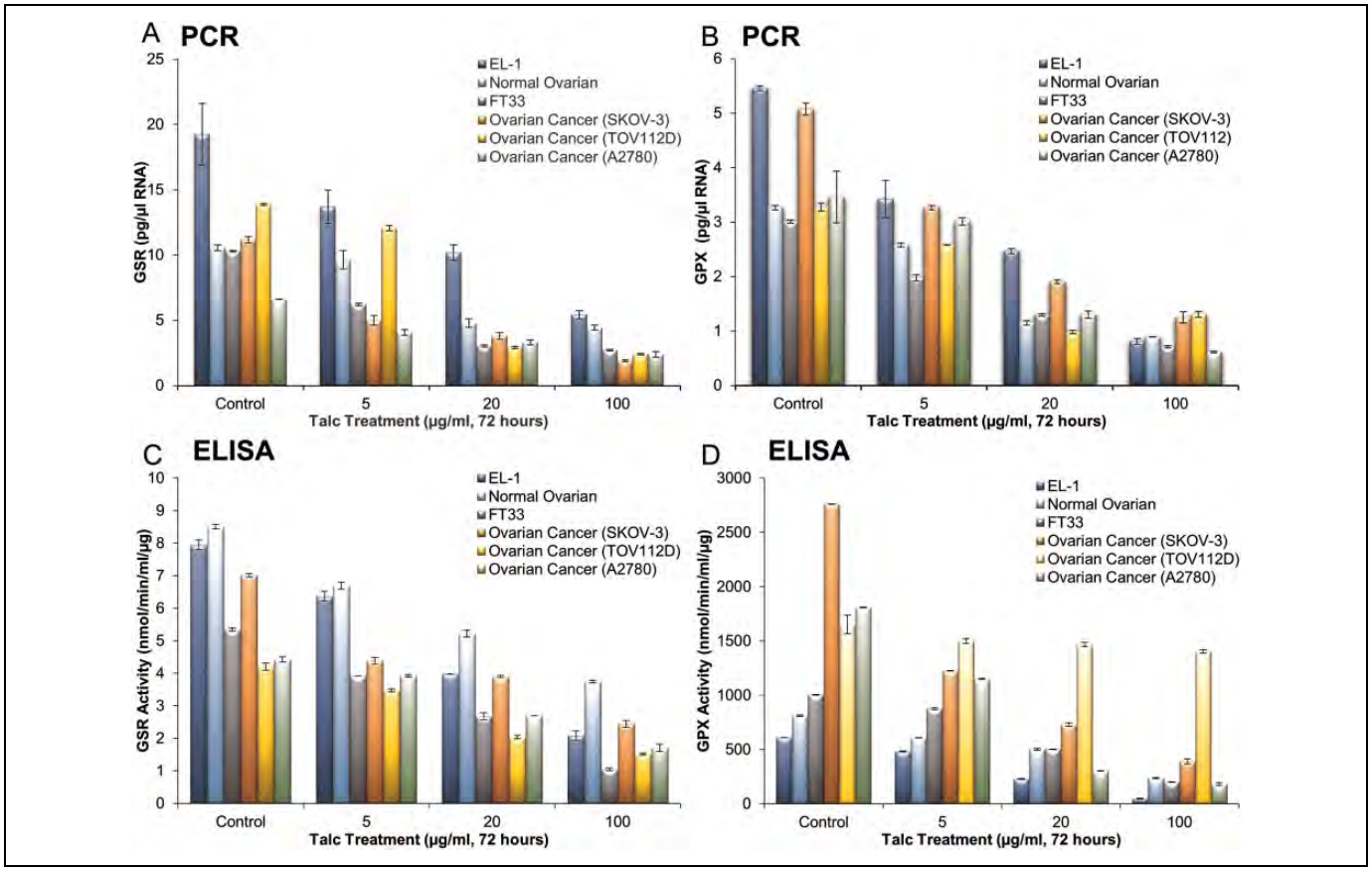

Figure 3. Decreased expression and activity of key antioxidant enzymes, GSR and GPX. The mRNA (real time RT PCR) and protein/activity levels (ELISA) of GSR (A and C) and GPX (B and D) were determined in macrophages (EL 1), human primary ovarian epithelial cells (normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV 3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 72 hours. Experiments were performed in triplicate. Expression is depicted as the mean, with error bars representing standard deviation. All changes in response to talc treatment were significant ($P < .05$) in all cells and in all doses as compared to controls. GSR indicates glutathione reductase; GPX, glutathione peroxidase; mRNA, messenger RNA; RT PCR, reverse transcription polymerase chain reaction; ELISA, enzyme linked immunosorbent assay.

fallopian tube, and macrophages. Oxidative stress has been implicated in the pathogenesis of ovarian cancer, specifically by increased expression of several key prooxidant enzymes such as iNOS, MPO, and NAD(P)H oxidase in EOC tissues and cells as compared to normal cells indicating an enhanced redox state, as we have recently demonstrated (Figure 7).[6] This redox state is further enhanced in chemoresistant EOC cells as evident by a further increase in iNOS and NO_2^-/NO_3^- and a decrease in GSR levels, suggesting a shift toward a prooxidant state.[6] Antioxidant enzymes, key regulators of cellular redox balance, are differentially expressed in various cancers, including ovarian.[6,14] Specifically, GPX expression is reduced in prostate, bladder, kidney, and estrogen receptor negative breast cancer cell lines, though GPX is increased in other cancerous tissues from breast.[14] Glutathione reductase levels, on the other hand, are elevated in lung cancer, although differentially expressed in breast and kidney cancer.[5,15] Similarly, CAT was decreased in breast, bladder, and lung cancer while increased in brain cancer.[16-18] Superoxide dismutase is expressed in lung, colorectal, gastric ovarian, and breast cancer, while decreased activity and expression have been reported in colorectal carcinomas and pancreatic cancer cells.[18-21] Collectively, this differential expression of antioxidants demonstrates the unique and complex redox microenvironment in cancer. Glutathione reductase is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to GSH. This enzyme is essential for the GSH redox cycle that maintains adequate levels of reduced cellular GSH. A high GSH to GSSG ratio is essential for protection against oxidative stress (Figure 5). Treatment with talc significantly reduced GSR in normal and cancer cells, altering the redox balance (Figure 3A and C). Likewise, GPX is an enzyme that detoxifies reactive electrophilic intermediates and thus plays an important role in protecting cells from cytotoxic and carcinogenic agents. Overexpression of GPX is triggered by exogenous chemical agents and reactive oxygen species and is thus thought to represent an adaptive response to stress.[15] Indeed, treatment of normal and cancer cells with talc significantly reduced GPX, which compromised the overall cell response to stress (Figure 3B and D).

Table 2. SNP Characteristics (A) and SNP Genotyping of Key Redox Enzymes in Untreated and Talc Treated (100 μg/mL) Human Primary Ovarian Epithelial Cells (Normal Ovarian), Human Ovarian Surface Epithelial Cells (HOSEpiC), Fallopian Tube (FT33), and Ovarian Cancer (A2780, SKOV 3, TOV112D) Cell Lines (B).

	Gene (rs Number)				
	CAT (rs769217)	*NOS*$_2$ (rs2297518)	*GSR* (rs8190955)	*GPX1* (rs3448)	*SOD3* (rs2536512)
A					
MAF	0.123	0.173	0.191	0.176	0.476
SNP	C 262T	C2087T	G201T	C 1040T	A377T
Chromosome location	11p13	17q11.2	8p12	3q21.31	4p15.2
Amino acid switch	Isoleucine to Threonine	Serine to Leucine	Unknown	Unknown	Alanine to threonine
Effect on activity	Decrease	Increase	Unknown	Unknown	Decrease
B					
A2780: Control	C/C	C/C	G/G	C/T	A/A
A2780: Talc	C/C	C/C	G/G	C/C	A/A
SKOV 3: Control	C/C	C/C	G/G	C/T	A/A
SKOV 3: Talc	C/C	T/T	G/G	C/C	A/A
TOV112D: Control	C/C	C/C	G/G	C/T	A/A
TOV112D: Talc	C/T	C/C	G/G	C/C	A/A
HOSEpiC: Control	C/C	C/C	G/G	C/T	A/A
HOSEpiC: Talc	C/T	T/T	G/G	C/T	A/A
FT33: Control	C/C	C/C	G/G	C/T	A/A
FT33: Talc	C/T	T/T	G/G	C/C	A/A
Normal ovarian: Control	C/C	C/C	G/G	C/T	A/A
Normal ovarian: Talc	C/T	T/T	G/G	C/C	A/A

Abbreviation: SNP, single-nucleotide polymorphism.

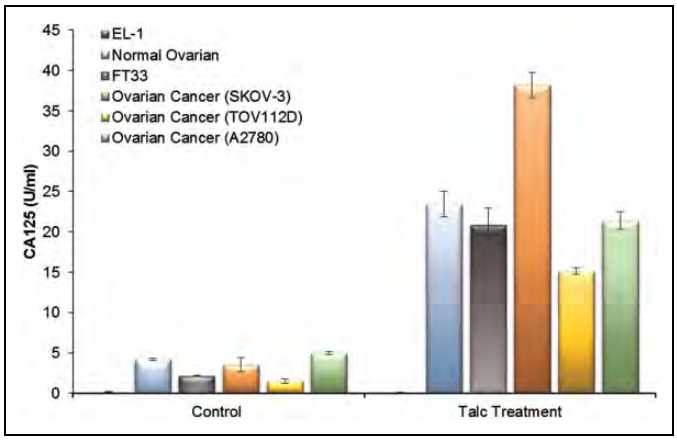

Figure 4. Increased CA 125 levels in response to talc treatment. The level of ovarian cancer biomarker CA 125 was determined by ELISA before and after 72 hours of talc treatment (100 μg/mL) in macro phages (EL 1), human primary ovarian epithelial cells (normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV 3, TOV112D, and A2780) cells. Experiments were performed in triplicate. Expression is depicted as the mean, with error bars representing standard deviation. All changes in response to talc treatment were significant ($P < .05$) in all cells as compared to controls. ELISA indicates enzyme linked immunosorbent assay.

We have previously reported that EOC cells manifest increased cell proliferations and decreased apoptosis.[6] In this study, we have shown that talc enhances cell proliferation and induces an inhibition in apoptosis in EOC cells, but more importantly in normal cells, suggesting talc is a stimulus to the development of the oncogenic phenotype. We also previously reported a cross talk between iNOS and MPO in ovarian cancer, which contributed to the lower apoptosis observed in ovarian cancer cells.[6,22] Myeloperoxidase, an abundant hemoprotein, previously known to be present solely in neutrophils and monocytes, is a key oxidant enzyme that utilizes NO produced by iNOS as a 1-electron substrate generating NO^+, a labile nitrosylating species.[6,23,24] We were the first to report that MPO was expressed by EOC cells and tissues and that silencing MPO gene expression utilizing MPO-specific siRNA induced apoptosis in EOC cells through a mechanism that involved the S-nitrosylation of caspase-3 by MPO.[22] Additionally, we have compelling evidence that MPO serves as a source of free iron under oxidative stress, where both NO^+ and superoxide are elevated.[6] Iron reacts with hydrogen peroxide (H_2O_2) and catalyzes the generation of highly reactive hydroxy radical ($HO^\bullet$), thereby increasing oxidative stress, which in turn increases free iron concentrations by the Fenton and Haber-Weiss reaction.[6,24] We have previously highlighted the potential benefits of the combination of serum MPO and free iron as biomarkers for early detection and prognosis of ovarian cancer.[25] Collectively, we now have substantial evidence demonstrating that altered oxidative stress may play a role in maintaining the oncogenic phenotype of EOC cells. Treatment of normal or ovarian cancer cells with talc resulted in a significant increase in MPO and iNOS, supporting the role of talc in the enhancement of a prooxidant state that is a major cause in the development and maintenance of the oncogenic phenotype (Figure 2).

Furthermore, CA-125, which exists as a membrane-bound and secreted protein in EOC cells, has been established as a

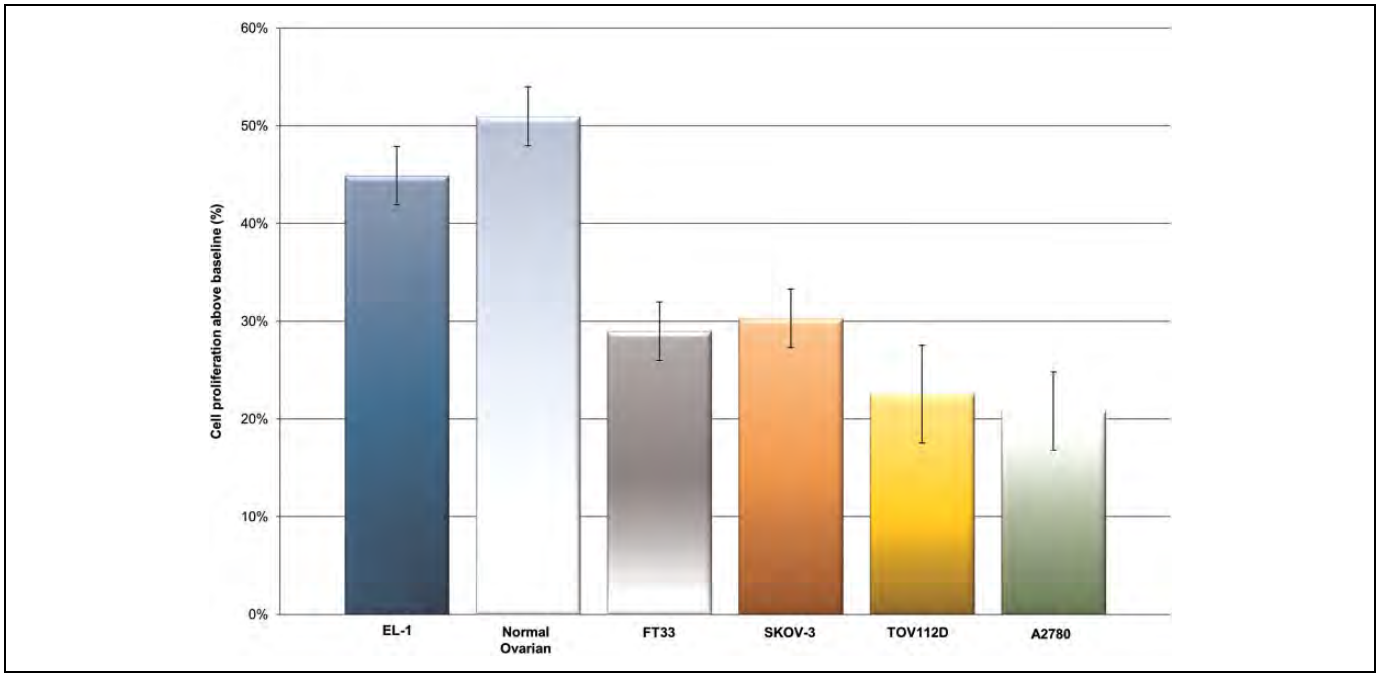

Figure 5. Increased cell proliferation in response to talc treatment. Cell proliferation was determined by MTT cell proliferation assay after 24 hours of talc treatment (100 μg/mL) in macrophages (EL 1), human primary ovarian epithelial cells (normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV 3, TOV112D, and A2780) cells. Experiments were performed in triplicate. Cell proliferation is depicted as the mean, with error bars representing standard deviation. All changes in response to talc treatment were significant ($P < .05$) in all cells as compared to controls.

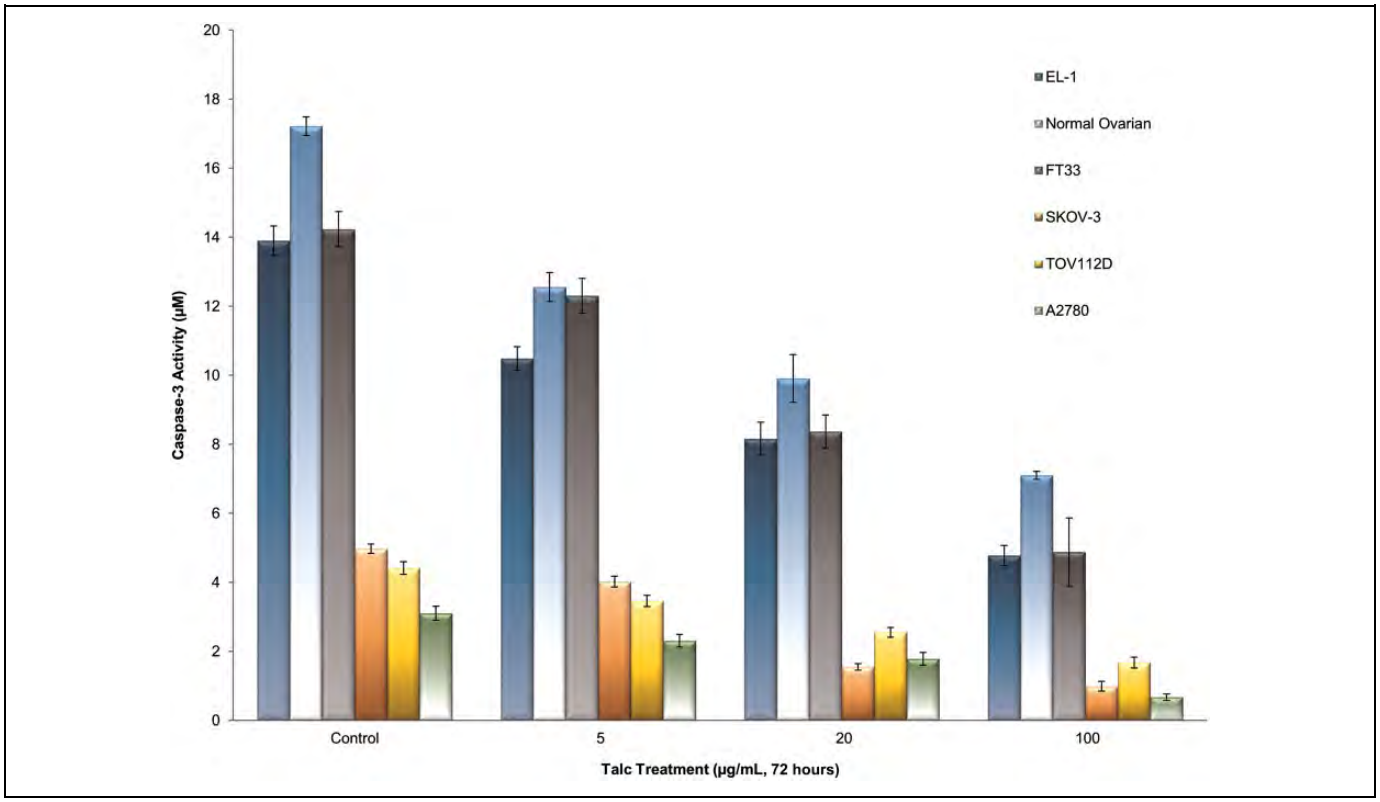

Figure 6. Decreased apoptosis in response to talc treatment. Caspase 3 activity was used to measure the degree of apoptosis in all cells. Caspase 3 activity assay was utilized to determine caspase 3 activity in macrophages (EL 1), human primary ovarian epithelial cells (normal ovarian), fallopian tube (FT33), and ovarian cancer (SKOV 3, TOV112D, and A2780) cell lines before and after treatment with various doses of talc over 72 hours. Experiments were performed in triplicate. Expression is depicted as the mean, with error bars representing standard error. All changes in response to talc treatment were significant ($P < .05$) in all cells and in all doses as compared to controls.

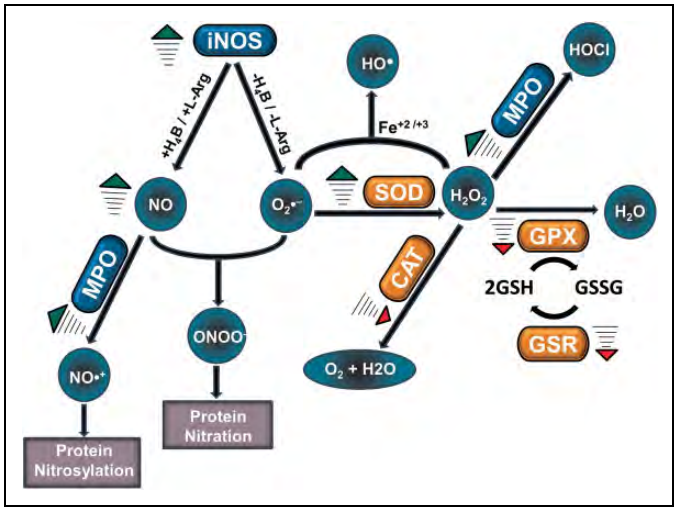

Figure 7. Epithelial ovarian cancer (EOC) cells have been reported to manifest a persistent prooxidant state as evident by the upregulation (green arrows) of key oxidants iNOS, NO, NO^+, $ONOO^-$, OH^-, O_2^+, and MPO (blue) and downregulation (red arrows) of key anti oxidants SOD, CAT, GPX, and GSR (orange). This redox state was also shown to be further enhanced in chemoresistant EOC cells. In this study, talcum powder altered the redox state, as indicated by the arrows, of both normal and EOC cells to create an enhanced proox idant state. iNOS indicates inducible nitric oxide synthase; MPO, mye loperoxidase; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; GSR, glutathione reductase.

biomarker for disease progression and response to treatment.[2] CA-125 expression was significantly increased from nearly undetectable levels in controls to values approaching clinical significance (35 U/mL in postmenopausal women[26]) in talc-treated cells (Figure 4, $P < .05$) without the physiologic effects on the tumor microenvironment one would expect to be present in the human body, thus highlighting the implications of the prooxidant states caused by talc alone.

To elucidate the mechanism by which talc alters the redox balance to favor a prooxidant state not only in ovarian cancer cells, but more importantly in normal cells, we have examined selected known gene mutations corresponding to SNPs known to be associated with altered enzymatic activity and increased cancer risk.[6,27] Our results show that the *CAT* SNP (rs769217) resulting in decreased enzymatic activity was induced in all normal cell lines tested and in TOV112D EOC lines, but was not detected in A2780 or SKOV-3 cell lines (Table 2). Nevertheless, our results confirm a decrease in CAT expression and enzymatic activity in all talc-treated cells (Figure 1), indicating the existence of other *CAT* SNPs. The *SOD3* (rs2536512) and *GSR* (rs8190955) SNP genotypes were not detected in any cell line, yet SOD3 and GSR activity and expression were decreased in all talc-treated cells, again suggesting the presence of other SNPs. Our results have also shown that all cells, except for HOSEpiC cells, manifest the SNP genotype of *GPX1* (C/T) before talc treatment. Intriguingly, talc treatment reversed this SNP genotype to the normal genotype (Table 2). Consistent with this finding, we have previously reported that acquisition of chemoresistance by ovarian cancer cells is associated with a switch from the *GPX1* SNP genotype to the normal *GPX1* genotype.[6] It is not understood why a *GPX1* SNP genotype predominates in untreated normal and ovarian cancer cells. Our results showed that talc treatment was associated with a genotype switch from common C/C genotype in *NOS2* in untreated cells to T/T, the SNP genotype, in talc-treated cells, except in A2780 and TOV112D (Table 2). Nevertheless, our results confirm an increase in iNOS expression and enzymatic activity in all talc-treated cells (Figure 2), again suggesting the existence of other *NOS2* SNPs. Collectively, these findings support the notion that talc treatment induced gene point mutations that happen to correspond to SNPs in locations with functional effects, thus altering overall redox balance for the initiation and development of ovarian cancer. Future studies examining such SNPs are important to fully elucidate a genotype switch mechanism induced by talc exposure.

In summary, this is the first study to clearly demonstrate that talc induces inflammation and alters the redox balance favoring a prooxidant state in normal and EOC cells. We have shown a dose-dependent significant increase in key prooxidants, iNOS, NO_2 /NO_3 , and MPO, and a concomitant decrease in key antioxidant enzymes, CAT, SOD, GPX, and GSR, in all talc-treated cells (both normal and ovarian cancer) compared to their controls. Additionally, there was a significant increase in CA-125 levels in all the talc-treated cells compared to their controls, except in macrophages. The mechanism by which talc alters the cellular redox and inflammatory balance involves the induction of specific mutations in key oxidant and antioxidant enzymes that correlate with alterations in their activities. The fact that these mutations happen to correspond to known SNPs of these enzymes indicate a genetic predisposition to developing ovarian cancer with genital talcum powder use.

Authors' Note

Ghassan M. Saed is also affiliated with Department of Gynecologic Oncology, Karmanos Cancer Institute, Detroit, MI, USA.

Acknowledgment

Special thanks to Imaan Singh for her technical contributions in acquiring the data and in development of graphics.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Dr. Saed has served as a paid consultant and expert witness in the talcum powder litigation.

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Exhibit 97

IN THE UNITED STATES DISTRICT COURT

FOR THE EASTERN DISTRICT OF NEW JERSEY

IN RE: JOHNSON & JOHNSON TALCUM POWDER PRODUCTS MARKETING, SALES PRACTICES, AND PRODUCTS LIABILITY LITIGATION

Case No. 16-2738 (FLW) (LHG)

THIS DOCUMENT RELATES TO ALL CASES

MDL Docket No. 2738

Friday, January 11, 2019

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The video deposition of SHAWN LEVY, Ph.D., taken pursuant to notice, was held at the Embassy Suites Huntsville, 850 Monroe Street S.W., Huntsville, Alabama, commencing at approximately 9:04 a.m., on the above date, before Lois Anne Robinson, Registered Diplomate Reporter, Certified Realtime Reporter, and Notary Public for the State of Alabama.

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COURT REPORTER

INDEX

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EXHIBITS

INDEX - (Continued)

I N D E X - (continued)

VIDEOGRAPHER:

We are now on the record. My name is Julie Robinson. I'm a videographer representing Golkow Litigation Services.

Today's date is January 11th, 2019, and the time is 9:04 a m.

This video deposition is being held in Huntsville, Alabama, in the matter of Johnson & Johnson Talcum Power Product Marketing, Sales Practices, and Products Liability Litigation, MDL Docket Number 2738.

The deponent is Dr. Shawn Levy.

Counsel will be noted on the stenographic record.

The court reporter is Lois Robinson, who will now swear in the witness.

SHAWN LEVY, Ph.D.,

the witness, after having first been duly sworn to tell the truth, the whole truth, and nothing but the truth, was examined and testified as follows:

EXAMINATION

BY MS. BROWN:

Q Good morning, Dr. Levy.

A Good morning.

Q My name is Alli Brown. I represent Johnson & Johnson, and I'll start with some questions for you here today.

Dr. Levy, have you ever been deposed before?

A Yes.

Q And tell me, how many times?

A In a setting like this, once.

Q Okay. What was the nature of that deposition?

A It was a patent litigation case.

Q Were you serving as an expert witness in that case?

A I was.

Q Were you hired by the plaintiffs or the defendants?

A The plaintiffs.

Q And, just generally, what were the issues in that case?

A It was entirely focused on evaluation of prior art in the genomic space.

Q And any time --

And do you remember the name of that

case, by the way?

A I don't. It was, gosh, twelve years ago or so.

Q I see.

Did that case go to trial?

A Not that I'm aware of.

Q Have you ever testified at trial?

A I have not.

Q Okay. And other than that one patent case you just described for us, were there other depositions that you've given?

A No.

Q And I think, when you started to answer the question in the beginning, you said "in a setting like this." Is there another time, in your mind, where you've given testimony under oath?

A No, not under oath. That's why I was --

So I've had a number of meetings, all limited to the patent space of mainly prior art discussions, where there's been representatives from both sides where we were having a discussion. But it wasn't a formal deposition

Page 10

with a court reporter, under oath, et cetera.
Q Understood.
So this would then be the second time you've been deposed in a setting like this.
A Correct.
Q Is that fair?
Okay. So a few ground rules that you may already be familiar with from your prior experience. First, we'll try not to speak over each other. Is that fair?
A That's fair.
Q That way, our court reporter can get down all my questions and all your answers. Okay?
A (Nods affirmatively.)
Q If you don't understand a question of mine, will you let me know?
A I will.
Q Okay. Try to verbalize your answers, too, so our court reporter can take them down. Okay?
A Understood.
Q Okay. If you need a break, let me know, and we'll be happy to accommodate you.

Page 11

Do you understand you're under oath here today, same as if you were in a court of law?
A I do.
Q Okay. I am --
And, before we get started, Doctor, I see you have a couple of items in front of you, and I want to identify what we have for the record.
To your right is an iPad that is showing the realtime of my questions and your answers. Will you be using that to assist you in your testimony here today?
A Yes.
Q Okay. In front of you you have a laptop computer.
A (Nods affirmatively.)
Q Will you be using that to assist you in your testimony?
A Yes.
Q And tell me, is this your laptop?
A It is not.
Q Okay. Whose laptop is it?
A The -- the attorneys I've been working

Page 12

with.
Q Okay. In front of you is the plaintiffs' lawyer's laptop. Is that right?
A That's right.
Q Okay. And what is contained on the plaintiffs' lawyer's laptop?
MS. O'DELL:
I think I'd probably be better to speak to it.
MS. BROWN:
No, no. Let's get it from the witness, and then if you want to make a statement for the record, of course.
Q Let's -- let's get your understanding of what's on this laptop in front of you.
A Other than what's on the USB drive that I've been using, I -- I don't have any knowledge of what's on it.
Q Okay. Do you know what's on the USB drive?
A I do.
Q What's that?
A It's a collection of literature cited in reliance literature list that -- from

Page 13

my -- from my report.
Q Did you put together the items that are contained on the USB drive that you have in front of you?
MS. O'DELL:
Object to the form.
A Yes.
MS. BROWN:
Q Is that your USB drive?
A No. I put together the list.
As far as who moved the files and organized the files on the USB, that, I don't know.
Q Okay. Are all of the files on that USB drive documents that you considered in connection with your opinion in this case?
A They are.
Q Any other materials in front of you that you'll be using to assist you in your testimony here today?
A There's a -- I have a hard copy of my report.
Q Did you prepare that hard copy binder?
A No.

Page 14

Q Who -- who did?
A My -- the -- the attorneys I've been working with. So I -- they -- they provided the printout and the nice binder that it's in.
Q Okay. Did you, Doctor, make any notes on the report that you have in front of you?
A No.
Q Okay. I'm gonna hand you what we have marked as Exhibit 1 to your deposition, which is a notice of your deposition.
(DEPOSITION EXHIBIT NUMBER 1 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q And I'll ask, is this something that you have ever seen before?
A Yes.
Q When did you see it?
A I'd have to review my email, but it was some -- sometime ago, some weeks ago.
Q Okay. Have you brought any --
And you understand that this Notice of Deposition that we've marked as Exhibit 1 requests that you bring certain documents with you here today?

Page 15

A Yes.
Q Okay.
MS. O'DELL:
Let me just insert for the record, we've objected to certain requests contained in the notice, and objections have been served, and materials have been brought to this deposition consistent with those objections.
MS. BROWN:
And we are in receipt of your objections.
Q And your counsel for the plaintiffs represented that some materials have been brought to the deposition. Do you have any materials with you responsive to this notice?
A Well --
MS. O'DELL:
I'll provide to you invoices that are responsive to the Notice, and there are materials that Dr. Levy has seen since his report was served, and -- and those are copies.
MS. BROWN:
Thank you, counsel.
Q So, Doctor, let's start --

Page 16

Thank you.
-- by marking these, and I'll ask you some questions about what we have.
(DEPOSITION EXHIBIT NUMBER 3 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q I'll mark as Exhibit 3 to your deposition two invoices counsel for plaintiffs just handed me, one dated May 2nd, 2018, and the other dated January 8th, 2019. And we only have one copy, so let me hand it to you and ask you, are these invoices that you created, Doctor?
A They are.
Q Okay. And I want to take that back for one second.
Looks like the first entry on your invoice is dated May 16th, 2017. Does that sound right to you?
A That sounds right.
Q When were you first approached about an involvement in this case?
A Earlier in 2017.
Q Okay. And who approached you?
A Leigh and Jennifer. I'd have to verify

Page 17

in my email whom I may have heard from first.
Q Okay. And Leigh and Jennifer are counsel for plaintiffs in this litigation; is that right?
A That's right.
Q And did they -- had you known them prior to receiving contact early in 2017 --
A No.
Q -- from plaintiffs' lawyers?
A I -- I did not know them.
Q Did they call you at your place of business?
A I believe the first contact was email. But, ultimately, yes.
Q Okay. And was there any connection, meaning did someone refer the plaintiffs' lawyers to you, or do you know?
A I don't know.
Q Do you have any idea how the plaintiffs' lawyers found you?
A I do not.
Q Okay. It looks like, Doctor, that these two invoices have a total of 33 hours. Does that sound right to you?

Page 18

A It does.
Q Looks like something's blacked out on the second page of the invoices. Do you know what that is?
MS. O'DELL:
I'll just say that redactions were made by counsel. They referenced the subject matter of conversations between Dr. Levy and counsel, and those have been redacted because of work product privilege.
MS. BROWN:
Okay.
Q Is it fair, Doctor, that you've spent a total of 33 hours forming your opinions in this case?
A That's fair.
Q Okay. Do you have any additional invoices that you plan to submit to the lawyers for the plaintiffs?
A Yes.
Q Okay. And can you ballpark for me how much additional time you've spent since the last entry here, which appears to be December 12th, 2018?

Page 19

A There's probably another -- not including this morning -- roughly 15 hours.
Okay. I'll hand you, Doctor, what we have marked as Exhibit 4 to your deposition. This is another document counsel for the plaintiffs just handed me.
(DEPOSITION EXHIBIT NUMBER 4 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q Would you identify that for the record, please.
A This is a printed copy from a website from the government of Canada discussing their draft screening assessment of talc.
Q Okay. Is that something you've seen before today?
A Yes.
Q When did you see it first?
A Sometime in December.
Q Did the lawyers for plaintiffs give it to you?
A They did.
Q Okay. Your report in this case --
Can I have that back?

Page 20

Your report in this case was served in November of 2018; correct?
A Correct.
Q Fair to say, then, that Exhibit 4, which you saw for the first time in December of 2018, did not inform the opinions contained in your report?
A That's correct.
Q Okay. Did the -- does Exhibit 4 contain any information regarding chronic inflammation as the proposed mechanism of ovarian cancer induced by talc?
A I don't believe it does. I'd have to review -- take a look at it to be sure.
MS. O'DELL:
And if you need to look at it, I'm sure counsel will hand it to you.
MS. BROWN:
Q I'm handing you, Doctor --
MS. O'DELL:
Excuse me. If you need to look at it to answer that question, you may.
A To be sure I'm accurate in my answer, I'd like to take a look at that.

Page 21

MS. BROWN:
Q Sure. Sitting here --
Hold on.
Sitting here today, you're not aware if Exhibit 4 contains any information regarding the proposed mechanism of chronic inflammation as a cause for ovarian cancer?
MS. O'DELL:
Object to the question.
If you need to see the document, Doctor, you may ask for it.
A Yeah. I'm not -- I'm not able to answer it accurately without seeing the document.
(DEPOSITION EXHIBIT NUMBER 5 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q Okay. Handing you what we've marked as Exhibit 5, would you tell me what that is, Doctor?
A This is another document from the government -- government of Canada discussing the potential risk of lung effects and ovarian cancer from talc.
Q Is Exhibit 5 a final document, do you

Page 22

know?
MS. O'DELL:
Object to the form.
A Yeah. That -- I don't -- I don't have the information available to answer that accurately.
MS. BROWN:
Q Have you seen Exhibit 5 prior to this morning?
A I have.
Q When did you first see Exhibit 5?
A Similar in time to the earlier report or this -- yes. Similar in time to the earlier -- to the same document from Exhibit 4.
Q To the best of your recollection, Doctor, you first saw Exhibit 5 after completing your report in this matter; is that right?
A That is right.
Q Fair to say, then, that Exhibit 5 did not inform the opinions contained in your MDL report?
A That's correct.
Q Handing you, Doctor, what we've marked as Exhibit 6 to your deposition, another document

Page 23

counsel provided, counsel for plaintiffs provided in response to your deposition notice.
(DEPOSITION EXHIBIT NUMBER 6 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q Would you identify for the record Exhibit 6?
A So this is a draft manuscript or preprint manuscript that's been submitted for peer review discussing the systematic review and meta-analysis of the association between perineal use of talc and risk of ovarian cancer.
Q Had you seen Exhibit 6 prior to this morning?
A Yes.
Q When did you first see Exhibit 6?
A It was in December as well.
Q Exhibit 6 did not inform your opinions in this matter. Fair?
A They did not inform the content of the report.
Q Have you reviewed and analyzed Exhibit 6 since December?
A I have.

Page 24

Q Does Exhibit 6 contain any information regarding the proposed mechanism of chronic inflammation?
A It does in reference, I believe. I'm reminding myself if -- if it shared the same materials that I had referenced in my report.
So, yes, it does.
Q Are you looking at a particular page, Doctor?
A I am.
Q And would you identify that for the record.
A I'm looking at page 23, beginning at line 220.
Q And what information does Exhibit 6 at page 23 contain regarding chronic inflammation?
A It discusses inflammation of the epithelial ovarian surfaces in animal models and provides two different references.
Q And were those references information you considered in forming your opinions in this case?
A Let me make sure of that.
Yes.

Page 25

Q And would you state what they are for the record, please?
A One reference is T.C. Hamilton, et al., The British Journal of Experimental Pathology, from 1984.
And the other reference is "The Pathology of Ovarian" -- "The Pathology of Ovarian Cancer Precursors," which is a review of R.E. Scully in the Journal of Cellular Biochemistry, and that is a supplement from 1995. The latter is not referenced in my report.
Q Have you reviewed the Scully paper in connection with your opinions in this matter?
A Not specifically, no.
Q You have, however, reviewed the Hamilton paper?
A Yes.
Q You would agree that the Hamilton paper does not show inflammation leading to neoplastic changes in animals?
MS. O'DELL:
Object to the form.
A I'd have to see the manu- -- or the manuscript to answer your specific question

Page 26

regarding neoplasm.
MS. BROWN:
Q Does the Hamilton paper support your view that chronic inflammation is a plausible mechanism for talc-induced ovarian cancer?
A It supports my opinion that inflammation is a component in the progression to ovarian cancer.
Q Is it your testimony that the Hamilton paper supports your opinion that chronic inflammation leads to neoplastic changes?
A No, not necessarily.
Q Okay. Tell me how it is that the Hamilton paper supports your opinion that chronic inflammation can cause ovarian cancer.
A Well, the -- so my opinion regarding -- that the role of inflammation in ovarian cancer is not based on a single study, particularly one that is now approaching or is now over 30 years old.
Q Okay. Does --
A So it's a -- I reviewed the -- that paper as well as a large number or the totality of the available evidence stretching across many

Page 27

years to develop the opinion that's represented in my report.
Q Sure.
A And to that opinion is -- no one study or one singular piece of information is the basis of that opinion.
Q Okay. But, you know, having reviewed Hamilton, that what Hamilton shows is that the inflammation they saw in the animals was not associated with neoplastic changes. Right?
MS. O'DELL:
Excuse me.
Doctor, if you'd like to -- to pull up Hamilton, you may do that.
MS. BROWN:
Q And we'll certainly give you time to do that, Doctor.
Sitting here today, do you recall that to be the conclusion of Hamilton?
MS. O'DELL:
Object to the form.
You don't -- if you need to see the --
MS. BROWN:
Counsel --

Page 28

MS. O'DELL:
-- paper in order to answer the question --
MS. BROWN:
Counsel --
MS. O'DELL:
-- you may do that.
MS. BROWN:
Counsel, he is absolutely entitled to get the paper. We're going to do that.
Q Sitting here today, do you recall --
MS. O'DELL:
But he is not --
MS. BROWN:
It's a fair question.
MS. O'DELL:
Is it not a fair question.
MS. BROWN:
I'm not gonna --
MS. O'DELL:
He's asking --
MS. BROWN:
-- do this with you.
MS. O'DELL:

Page 29

Yes, you are. If he's asked to see the paper, he gets to look at the paper. Because this is not a situation where you can say, "Oh, I'll show it to you later," ask all these questions, try to get him to answer when he said I want to see the paper and review it. That's the way this works.
MS. BROWN:
Q Dr. Levy, can you answer the question without looking at the paper?
MS. O'DELL:
Would you repeat the question just to make sure we've got it?
MS. BROWN:
Yes. Would you please keep your objections to form in accordance with the federal rules?
MS. O'DELL:
My objections have been in accordance with the federal rules.
MS. BROWN:
Q Dr. Levy, my question to you was whether the Hamilton paper, the findings of the Hamilton paper show that chronic inflammation led

Page 30

to neoplastic changes. Do you recall that question?
A I do recall the question.
Q Can you answer that question without looking at the paper?
A I would need to look at the paper to accurately answer your question.
Q Absolutely. Do you have a copy on your computer?
A I do.
Q Okay. We'll mark it, so we're all on the same page, as Exhibit 7.
(DEPOSITION EXHIBIT NUMBER 7 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q Here's a hard copy, Doctor, if that assists you.
Doctor, looking at the Hamilton article that you have in front of you, does that refresh you that the authors found no association between the talc-induced changes and neoplasm?
A No. Their -- their conclusions were that the talc-induced changes -- specifically fibrosis and the papillary changes -- did not

Page 31

appear to be a reaction to talc, but they -- I don't see the specific inclusion that you asked in the question regarding neoplasm.
Q I'm looking at page 103, Doctor, the first full paragraph that begins "no evidence."
You with me?
A One moment. "No evidence of cellular," that paragraph?
Q Yes.
And, for the record, that paragraph reads, "No evidence of cellular atypia or mitotic activity was seen in the nonpapillary areas of the surface epithelium of the injected ovaries and in no ovary was there any evidence of frank neoplasia."
Correct?
A It does read that way, yes.
Q And that was a conclusion of the Hamilton article. Correct?
MS. O'DELL:
Object to the form.
A That was an observation of the Hamilton article.
MS. BROWN:

Page 32

Q The Hamilton article does not support the theory that chronic inflammation leads to neoplastic changes in the ovary. Fair?
MS. O'DELL:
Object to the form.
A The Hamilton article looked at an interval of one month, eighteen months, in a rat model. And, so, in the constraints of that particular experimental design and given the state of the art of the technology at the time, the authors did not conclude of a significant progression of ovarian cancer. But there's clearly limitations in both their experimental design and time course of the study to draw wide conclusions.
MS. BROWN:
Q The conclusions of the Hamilton article, Dr. Levy, do not support the hypothesis that chronic inflammation from talcum powder causes ovarian cancer. Would you agree?
A I would not.
Q The authors did not find that the inflammation seen in Hamilton led to neoplastic changes. True?

Page 33

A The authors did not report observing neoplastic change over the time course of the given study.
Q Doctor, I'm handing you the report that you've served in this case, which we'll mark as Exhibit 2.
(DEPOSITION EXHIBIT NUMBER 2 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q And I'd like you to -- I'd like to direct you to page 14. I'd like to direct your attention to the last paragraph of -- the last sentence -- excuse me -- of the second full paragraph that begins "additional studies."
Do you see that sentence, Doctor?
A What's the beginning of that paragraph so I make sure I'm looking at the right one?
Q Sure. I'd like to direct you on page 14 of your report to the second full paragraph that begins "In addition to epidemiologic evidence."
Do you see that?
A I do.
Q The last paragraph, or the last

Page 34

sentence of that paragraph in your report reads, "Additional studies have also shown the effects of talc on the immune response."
Do you see that sentence?
A I do.
Q And you cite the Hamilton article for that proposition that we were just reviewing?
A Uh-huh.
Q True?
A True.
Q And the talc effects on the immune response that were shown in Hamilton were not effects that the authors observed led to neoplastic changes. Correct?
MS. O'DELL:
Object to the form.
A I'm sorry. I'm not sure I understand your question.
MS. BROWN:
Q Sure.
A Are you asking, if I could clarify, are you -- are you asking if Hamilton is an appropriate reference for the effects of talc on the immune response or are you asking if

Page 35

Hamilton's an appropriate reference for something else?
Q In your report, you state that studies, such as Hamilton, have shown effects of talc on the immune response. Correct?
A That is correct.
Q And you said Hamilton as one of the articles that supports that proposition. True?
A Of the immune response, that's true.
Q Okay. The immune response that was observed in Hamilton was not an immune response that led to cancer. Right?
A As -- as I stated earlier, on the time course of the Hamilton study, the authors did not report specifically to neoplastic change in the rat or conclude or make that conclusion, nor did they conclude that that was not a possibility either.
Q And on page 14 of your report you have two additional cites for that proposition; correct?
A Correct.
Q And you know, Doctor, that neither of those cites, Keskin or NTP, support the

Page 36

hypothesis that chronic inflammation leads to cancer in animals. Right?
A The --
MS. O'DELL:
Object to the form.
A The -- those two references were not included in the report to provide the opinion or conclusions that you just described.
MS. BROWN:
Q Because you know, Doctor, that there's not a single animal study that shows that talc causes changes in animals that leads to cancer; right?
MS. O'DELL:
Object to the form.
A Could you -- could you phrase that question again? Sorry.
MS. BROWN:
Q There is not a single animal study, Doctor, that supports the opinion that chronic inflammation caused by talc causes ovarian cancer. Is that correct?
MS. O'DELL:
Object to the form.

Page 37

A In my review of the literature, there are a number of animal studies that support the opinions in the report regarding the biological plausibility of talc leading to or contributing to neoplastic change.
MS. BROWN:
Q Are you aware of any animal studies, Doctor, that show talc causing chronic inflammation in animals that leads to neoplastic or cancerous changes in the animals?
MS. O'DELL:
Object to the form. Compound.
A There is one 1971 study that I'm aware of. I would have to review to remember the author. That was an earlier seminal -- or a earlier study that described the role of talcum powder and the inflammatory change within the ovary.
MS. BROWN:
Q Who's the author of that study, Doctor?
A I'm trying to think of where I have that reference.
Q Why don't we put that to the side and at a break we'll see if we can find that article

Page 38

and then we can take a look at it. Okay?
A Uh-huh.
Q Okay. Getting back, then, Doctor, to what we had marked as Exhibit 6, which is the Taher paper, fair to say you reviewed that paper after your report was submitted in this case?
A Yes.
Q Okay. And did you notice throughout Taher's paper he makes reference to a number of supplemental materials?
A Not specifically.
Q Are you in receipt from plaintiffs' counsel of those supplemental materials?
A I'd have to -- you'd have to give me a specific example, and I would be able to answer you.
Q So, throughout the paper, the authors make reference to a set of supplemental materials that support their opinions. Do you recall that?
A I certainly recall the reference materials to support their opinion. Whether they were supplemental or otherwise, that doesn't stand out to me.
Q Okay. And I'm not trying to be tricky.

Page 39

I just want to know if you have those materials, and, if so, I'm gonna request production of them.
A No. I -- I -- I don't believe that I have the full list of reference -- of literature cited from that -- from this paper --
Q Okay.
A -- now --
Q Now, Taher --
A -- but I'd have to check.
Q Sorry.
The Taher paper did not inform your -- the opinions contained in your report dated November of 2018; correct?
A Correct, as written.
Q Okay. Are there any additional documents that either you or your counsel have brought with you here today in response to Exhibit 1, the Notice of Deposition?
A So I'm not sure how to answer that accurately, but I would say there's a -- I've been provided with -- since the completion of my report, I've been provided with reports from other experts in the -- in the case.
Q Okay.

Page 40

A And I have those on the -- available electronically.
Q Okay. Were you provided with completed versions of all the plaintiff experts in the MDL proceeding?
A I can't speak to whether it was all, but I have been provided with several.
Q Will you list for me the expert reports you've been provided with?
A Sure.
Q Thank you.
A There are four on -- on this drive, three -- I'm sorry. Two. Crowley and Longo.
Q Two reports from Dr. Crowley and two reports from Dr. Longo?
MS. O'DELL:
I don't think that's what he said.
A No. I think there are two, two expert reports, one from Dr. Crowley and one from Dr. Longo.
MS. BROWN:
Q Okay. And the date of the Crowley report, please?
A The -- according to the file, the

Page 41

date -- the modified date is November 28, 2018.
Q And --
A Whether that was the written date, I -- I don't know.
Q And the Longo report, do you know the date of that?
A It is listed as August 2nd, 2017, in the title. And then there's a -- sorry. There's a second Longo report, 2018, which has a November 28, 2018, date. So my -- my apologies. To correct, there are two expert reports from Dr. Longo.
Q Got it.
MS. O'DELL:
So when you were talking about --
MS. BROWN:
Counsel, no. Huh-uh. No. We -- I'm gonna ask questions, and he's gonna answer. We are not going to have you testify. You are not to testify about the expert reports.
MS. O'DELL:
I'm not gonna --
You asked him what the date of the report was.

Page 42

MS. BROWN:
He -- then he will answer, counsel. You can't testify.
MS. O'DELL:
He gave you the date of the file -- the file date --
MS. BROWN:
That's fine.
MS. O'DELL:
-- not the date --
MS. BROWN:
On redirect, you are welcome to clean up whatever you need to. But we're not gonna have your testimony on the record about dates of expert reports.
A So, looking at the report itself, the date of the Longo report is November 14th, 2018.
MS. BROWN:
Q And were you provided --
A The -- would you like the date of the earlier report?
Q That would be terrific.
A It's August 2nd, 2017.
Q Great.

Page 43

Were you provided the two Longo reports and the Dr. Crowley report by plaintiffs' counsel?
A Yes.
Q Do you recall when?
A Not specifically. It was, obviously, by their date, sometime after their completion. So the Crowley report and the later 2018 Longo report were sometime in November or December 2018.
There's -- I've also had an opportunity to review a number of -- several other expert reports which are not with me today.
Q Do you have a listing of the additional expert reports you were provided with?
A I'd have to -- I could certainly -- I'd have to provide it. I don't, off the top of my head, recall all of them. There was probably approximately a dozen.
Q Were all of the plaintiff expert reports sent to you at once?
MS. O'DELL:
Object to the form.
A I'm not -- I'm not certain.

Page 44

MS. BROWN:
Q How did you receive them? Was it email or hard copy?
A Neither. They were made available through a shared storage.
Q And would you have received an email alerting you to their existence on a shared file?
MS. O'DELL:
Dr. Levy, communications between counsel are -- are subject to the work product privilege.
So to the degree you're asking him to convey what was in a communication, then I'll object to that and instruct you not to discuss communications between counsel.
MS. BROWN:
Q Which the question does not ask for, Doctor.
MS. O'DELL:
I believe it does.
MS. BROWN:
Q Here's what I want to know. Did you rely on any other expert reports in forming your opinions in this case?

Page 45

A To -- to my -- the content of my report, no.
Q Did you receive the Crowley and two Longo reports after you had already completed your report in this case?
MS. O'DELL:
Object to the form.
A No. There was -- if I recall -- and the -- at least the earlier Longo report -- and I'd have to review the specifics -- at least the earlier Longo report was reviewed and was included in the content in the report.
And I would have to -- since the later Longo report and then the final version of this report were quite close together, I don't recall if they overlapped or not. I'd have to review the -- which references I used in here, which will just take a moment.
So, yes, the -- I did include both Longo reports.
Q The second Longo report was finalized two days prior to your report. Is that right?
A Finalized, yes.
Q Did you see a draft of Longo's 2018

Page 46

report?
A Yes. And the --
Q And did you --
A And as to when I saw the draft, I believe it was -- and it was sometime in the fall and/or when reports were being revised and expanded as more literature became available.
Q Prior to Longo finalizing and signing his expert report in the MDL, you had access to a draft of that report; is that right?
MS. O'DELL:
Object to the form.
A I can't speak to -- to that accurately.
MS. BROWN:
Q I thought you just testified you saw a version of the Longo 2018 report that was not final. Is that correct?
MS. O'DELL:
Object to the form.
A I'd have to -- I'd have to review my -- the -- the literature that I used for the report to accurately answer your question.
MS. BROWN:
Q Well, your report doesn't say a draft,

Page 47

and I'm wondering if you ever saw a non-finalized copy of the Longo report.
A I didn't have an opportunity to compare the finalized Longo report to a -- what may be a draft or not to accurately answer your question if I saw a draft that was substantially different than what's referenced as the final.
Q There were two days between Longo serving his report and you serving your report. Does that help orient you as to whether you saw a draft or you saw the final version?
A Certainly possible I saw the final version.
Q How many hours did you spend on your report in this case, Doctor?
A The initial draft of the report? The initial writing of the report?
Q In total, how many hours did you spend writing your report?
A It was 20 hours initially, and then it would be -- it would be difficult to provide an accurate answer for the rest of that. I would say an additional few hours that I counted as revision.

Page 48

Q Did you type the expert report that we've marked as Exhibit 2 yourself?
A I did.
Q Did you write all contents of Exhibit 2 yourself?
A I did.
Q Were there parts of your report that you lifted from other published articles?
MS. O'DELL:
Object to the form.
A Could you describe "lifted"?
MS. BROWN:
Q Did you take the words of other authors and put them in your expert report as Exhibit 2?
MS. O'DELL:
Object to the form.
A No. My -- my -- so my report is a review of the available literature at the time that the report was being developed. So, as such, it describes that -- that literature.
As far as did I specifically copy words from other reports, no.
MS. BROWN:
Q Did you work with another plaintiff

Page 49

expert on the report that we've marked as Exhibit 2?
A I did not.
Q Do you know who Dr. Zelikoff is?
A The name's not familiar to me.
Q Did you review a draft of Dr. Zelikoff's report before submitting your own?
A I did not.
Q Do you think that --
A Not that I'm aware of.
Q Do you have any explanation as to why a paragraph in your report is the same as a paragraph in Dr. Zelikoff's report?
MS. O'DELL:
Object to the form.
A I -- without knowing -- without seeing the paragraph in both reports would be -- I can't comment.
MS. BROWN:
Q Let's mark as Exhibit 8 the expert report of Dr. Judith Zelikoff, Ph.D.
(DEPOSITION EXHIBIT NUMBER 8 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:

Page 50

Q Is this something you've seen --
Oh, sorry. Can I --
It's okay, actually. It will flag it for you?
Is this a report that you've seen before, Doctor?
A I'll have to see it before I answer.
Q I'm handing you what we've marked as Exhibit 8, which is the expert report of Dr. Judith Zelikoff. Is this one of the reports that you reviewed prior -- you reviewed at all?
A I would have -- I would actually have to review my -- the literature that I reviewed in -- the totality of the literature that I reviewed, which I could answer that after a break, if necessary. But I don't recall, specifically recall, this report under Dr. Zelikoff's name. But it is certainly possible that I may have seen...
Q Let's look at page 5 of your report, Doctor.
A Okay.
Q And why don't you put that side by side with page 20 of Dr. Zelikoff's report. And the

Page 51

paragraph in Dr. Zelikoff's report that I want to direct you to is the first full paragraph on page 20 that begins "Genetic mutations."
Do you see that?
A I do.
Q And the paragraph of your report I want to direct you to is the paragraph on page 5 that begins "Both inherited."
Do you see that?
A I do.
Q Okay. The first sentence of that paragraph in your report reads, "Both inherited and acquired gene -- and acquired gene mutations work together to cause cancer."
Do you see that?
A I do.
Q The third sentence of the paragraph I directed you to in Dr. Zelikoff's report is identical and reads, "Both inherited and acquired gene mutations work together to cause cancer."
Do you see that?
A I do.
Q Those two sentences are exactly the same, are they not?

Page 52

A They are --
Q The next sentence --
A Just one moment, please. I'm just making sure. Your question was are they exactly the same, and I'm just confirming if they're exactly the same.
So, yes, I agree they're exactly the same.
Q You have reviewed them and satisfied yourself that that -- those two sentences are exactly the same; correct?
MS. O'DELL:
Object to the form.
A There's a single sentence in each report that is exactly the same. But important to comment that this single sentence is a -- is a basic biological premise of cancer, and, so, there's no surprise that two expert witnesses offering opinions on the role of -- or the biological plausibility or mechanisms of development of cancer would introduce a fundamental premise in the same manner.
MS. BROWN:
Q No surprise that you experts would have

Page 53

one sentence that's the same? Is that what you're saying?
MS. O'DELL:
Objection. That's not what he said. Misrepresents his testimony.
A I'm saying that both would -- both reports detail a fundamental aspect as they would -- based on the current understanding of the -- that both inherited and acquired gene mutations work in concert to cause cancer.
MS. BROWN:
Q Look at the next sentence on page 20 of Dr. Zelikoff's report. It reads as follows: "Even if one has inherited a genetic mutation that predisposes one to cancer," comma, "that doesn't mean he or she is certain to get cancer."
Did I read that correctly?
A You did.
Q And let's go back to page 5 of your report. Skip ahead, if you would -- one, two, three -- four sentences to where you were and find the sentence that begins "Even."
Are you with me?
A I am.

Page 54

Q And your report at page 5 reads, "Even if one has inherited a genetic mutation that predisposes one to cancer," comma, "that doesn't mean he or she is certain to get cancer."
Did I read that correctly?
A You did.
Q That's the exact same sentence we just read in Dr. Zelikoff's report; correct?
A It is.
Q So now we have two sentences that are exactly the same in your report and Dr. Zelikoff's report. Correct?
MS. O'DELL:
Object to the form.
A You have two sentences that are written the same but certainly not in precisely the same context or organization in the total report.
MS. BROWN:
Q We have two sentences that are word-for-word identical in two of the plaintiffs' expert reports in this litigation. Is that fair?
MS. O'DELL:
Objection. Asked and answered.
A So reading your earlier question, you

Page 55

asked, "Is that the same exact sentence we just read in Dr. Zelikoff's report; correct?" And my answer was "It is." And it remains the same.
Q Let's keep going.
Next sentence, at page 20 in Dr. Zelikoff's report, states as follows: "Rather," comma, "one or more additional gene mutations may be needed to cause cancer."
Did I read that correctly?
A You did.
Q Let's go back to page 4 -- excuse me -- page 5 of your report where we just were. And you write: "Rather," comma, "one or more additional gene mutations may be needed to cause cancer." Correct?
A Correct.
Q That is the identical sentence from Dr. Zelikoff's report. Correct?
A Starting with "Rather, one or more additional gene mutations may be needed to cause cancer."
Yes, correct.
Q So we now have identified three sentences in Dr. Zelikoff's report that are

Page 56

identical to your report; correct?
A We have.
Q Do you have any explanation for why that would be?
A I do.
Q What's that?
A That these -- each of these sentences are describing basic introductory information around the relationship between cancer and genetic mutation.
Q And each of you described it with the exact same words?
A Apparently so.
Q Let's keep going.
Page 20 of Dr. Zelikoff's report, picking up where we left off, Dr. Zelikoff writes: "The inherited gene mutation could instead make one more likely to develop cancer when exposed to certain cancer-causing substances."
Do you see that?
A I do.
Q And let's go back to where we were in your report, on page 5. "The inherited gene

Page 57

mutation could instead make one more likely to develop cancer when exposed to a certain cancer-causing substance."
Do you see that?
A I do.
Q And other than the tense in that last sentence, they, too, are identical. Correct?
A So they're -- they're certainly similar sentences, but that -- I believe the tense is an important difference between them.
Again, as I stated, that these are introductory and fundamental perspectives on cancer and that, in this case, two expert witnesses have summarized those things in a similar fashion.
Q It doesn't strike you as odd that four sentences are identical from two expert reports?
MS. O'DELL:
Object to the form.
A Four sentences are not identical.
MS. BROWN:
Q There's one small change in a tense. That's it. Right, Doctor?
MS. O'DELL:

Page 58

Object to the form.
A There -- there are -- there are three sentences which are, when considered individually, they are the same words. When you consider the -- now the group of those four sentences together between the two reports, they are clearly different organization with significantly more information between those identical sentences in one or the other.
So the suggestion that they were -- one report was copied into the other, I would say it is equally interesting that they are more different than they are alike, other than the wording of three sentences.
MS. BROWN:
Q Did someone other than you write the sentences we've just been looking at in your report?
A No.
Q Did you consult the Mayo Clinic's website in connection with writing your report?
A I don't believe so.
Q Do you consider the Mayo Clinic's website to be authoritative -- an authoritative

Page 59

source, in your view?
MS. O'DELL:
Object to the form.
A I have no basis for that opinion. I -- I haven't reviewed the Mayo Clinic website to determine that.
(DEPOSITION EXHIBIT NUMBER 9 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q Handing you, Doctor, what we've marked as Exhibit 9 to your deposition, which is a printout from the Mayo Clinic website entitled "Cancer."
A Uh-huh.
Q I'll hand it to you. And let me know if this is something that you've ever seen before.
A Not that I recall.
Q Did you take any language from the Mayo Clinic website to use in your report?
A No.
Q Let's take a -- I want you to put the Mayo Clinic, which we've marked as Exhibit 9 --
A Uh-huh.

Page 60

Q -- next to your report, which remains Exhibit 2. And I will direct you to the second page of the Mayo Clinic printout, the section titled "Causes."
Are you with me?
A Second page.
Q Double-sided. Flip it over.
A Yes.
Q Okay. And I'll direct you to page 3 of your report entitled "The Role of Gene Mutations in the Development of Cancer."
A Uh-huh.
Q Starting with Exhibit 9, the Mayo Clinic website, under a section entitled "Causes," the Mayo Clinic writes, "Cancer is caused by changes" -- parentheses -- "(mutations) to the DNA within cells."
Do you see that?
A I do.
Q And, looking at page 3 of your report, Doctor, that same sentence or sentence fragment appears in the first sentence: "Cancer is caused by changes" -- parentheses -- "(mutations) to the DNA within cells."

Page 61

Correct?
MS. O'DELL:
Object to the form.
A Say your question again. Are you asking --
MS. BROWN:
Q It's the same; right, Doctor?
MS. O'DELL:
Object to the form.
A There are eight words or ten words that are the same in this first sentence, again, both describing some of the fundamental premise of cancer and its -- in its description.
MS. BROWN:
Q Let's go to the second sentence in the Mayo Clinic website, which reads, "The DNA inside a cell is packaged into a large number of individual genes, each of which contains a set of instructions telling the cell what functions to perform," comma, "as well as how to grow and divide."
Do you see that?
A I do.
Q And a nearly identical version of that

Page 62

sentence appears in your report at page 3 where you state, "The DNA that makes up our genetic code is organized into a large number of individual genes, each of which contains a specific subset of instructions telling the cell what functions to perform," comma, "as well as how to grow and divide."
Do you see that?
A I do.
Q Do you notice that nearly all the words are the same as the Mayo Clinic's?
MS. O'DELL:
Objection to form.
A I, again -- we -- we have another example of similar language describing introductory and fundamental aspects surrounding the basics of cancer biology.
MS. BROWN:
Q Back to the Mayo Clinic next sentence. Quote: "Errors in the instructions can cause the cell to stop its normal function and may allow a cell to become cancerous."
Do you see that?
A I do.

Page 63

Q Back to your report at page 3. An identical sentence: "Errors in the instruction can cause the cell to stop its normal function and may allow a cell to become cancerous."
Do you see that?
A I do.
Q Does that strike you as strange?
MS. O'DELL:
Object to the form.
A Strange in what way?
MS. BROWN:
Q That your expert report in this litigation contains identical sentences to the Mayo Clinic's website.
MS. O'DELL:
Objection. Misstates the report.
A I -- I don't find it surprising in the least.
MS. BROWN:
Q Let's turn to page 4 of your report, please. And I'll direct you to the final bullet on the same page of the Mayo Clinic website you were just looking at. The section of your report on page 4 I'd like to direct you to is the

Page 64

subparagraph titled "Loss of DNA Repair."
Are you with me?
A Yes.
Q I'm gonna read you two sentences from the Mayo Clinic. Tell me if I read them correctly.
"DNA repair genes look for errors in a cell's DNA and make corrections. A mutation in a DNA repair gene may mean that other errors aren't corrected, leading cells to become cancerous."
Do you see those two sentences, Doctor?
A I do.
Q Those are two sentences written by the folks who produce the Mayo Clinic's website; correct?
A I -- I have no knowledge of who wrote that.
Q The same two sentences appear in your report on page 4. Quote: "DNA repair genes look for errors in a cell's DNA and make corrections. A mutation in a DNA repair gene may mean that other errors aren't corrected, leading cells to become cancerous."
Do you see that?

Page 65

A I do.
Q Those two sentences are identical in the Mayo Clinic's website and your report. True?
MS. O'DELL:
Object to the form.
A Again, we have fund- -- basic information that provides an introductory description of the basics of cancer which is used as -- as an inform- -- informatory foundation for latter opinions in the report but is not germane to the -- to the opinion in my report.
And, again, as stated before, that succinct fundamental information regarding cancer biology in two sources that state things succinctly and clearly in layman's language are -- are similar or even identical, again, does not surprise me.
MS. BROWN:
Q We read at least four sentences that are identical to the Mayo Clinic. Would you agree?
MS. O'DELL:
Objection to form. The sentences are not identical.

Page 66

MS. BROWN:
Counsel, form.
A There are some similar -- there are some similarly stated sentences that you're -- that you've taken out of context in both cases to find them identical. So I -- I agree that they're identical, but, again, don't -- don't necessarily am surprised since I have no knowledge of where the information from the Mayo website was taken from.
MS. BROWN:
Q You agree a number of sentences in your report are identical to a number of sentences on the Mayo Clinic's website. True?
MS. O'DELL:
Object to the form.
A No. I agree that they're -- I don't agree. There are specific wordings that are the same.
MS. BROWN:
Q Doctor, do you not agree that a number of the sentences we just read are identical to a number of sentences that appear on the Mayo Clinic's website?

Page 67

MS. O'DELL:
Object to the form.
A I think we've -- we've specifically gone over those individually and answered those questions.
MS. BROWN:
Q And you'll agree the sentences are identical?
MS. O'DELL:
Object to the form.
A Again, I -- I've answered -- I've answered those when we went through them individually.
MS. BROWN:
Q Well, I want you to answer my question now.
You'll agree we've looked at a number of sentences that are identical in your report to the information on the Mayo Clinic's website; correct?
MS. O'DELL:
Object to the form. Misstates his testimony.
A I'd have to go back to the transcript

Page 68

from our conversation to comment on those.
MS. BROWN:
Q You have it right in front of you. We just looked at them.
A We did.
Q Right?
A Yes.
Q You recall reading a number of sentences in the Mayo Clinic website that match word for word a number of sentences in your report. True?
MS. O'DELL:
Object to the form.
A We've -- we've read information that is -- that is similar between the two documents. And, as answered, given the, again, basic fundamental introduction in lay language for these concepts, it is no surprise that it's the same.
MS. BROWN:
Q You're not surprised to find identical sentences in your report and Dr. Zelikoff's report?
A I'm not surprised.

Page 69

MS. O'DELL:
Object to the form.
MS. BROWN:
Q You are not surprised to find identical sentences in your report and the Mayo Clinic?
MS. O'DELL:
Objection to form. Asked and answered.
A No. I -- I've answered that.
MS. BROWN:
Q You need to answer it again.
Are you --
A I'm not surprised.
Q -- surprised?
Did you consult Wikipedia in writing your expert report?
A I don't recall.
Q Do you think it's possible you might have looked at Wikipedia when writing your expert report in this litigation?
A I've -- I've looked -- I've looked at a large number of sources in published literature and others.
Q Did one of those sources include Wikipedia?

Page 70

A I don't recall.
Q Do you consider Wikipedia to be a scientifically reliable source?
A What do you mean by scientifically reliable.
Q Do you understand the concept of scientific reliability when answering a scientific question?
MS. O'DELL:
Object to the form.
A Again, you'd have to -- that's -- you'd have to explain your -- what scientific reliability means in the context of your question.
MS. BROWN:
Q What does it mean to you?
A Scientific reliability? In general terms, it would mean information that comes from a peer-reviewed source.
Q And Wikipedia is not peer-reviewed; correct?
A Wikipedia generally reso- -- uses a -- is a summary of commonly -- at least in scientific terms, a number of peer-reviewed

Page 71

sources, but it is --
So from a true peer-review perspective, Wikipedia actually is peer-reviewed in the sense that anyone can contribute and edit the information in Wikipedia.
Q Including our kids; right?
MS. O'DELL:
Object to the form.
A Possible.
MS. BROWN:
Q Anyone in the world could edit a Wikipedia page. True?
A I believe so.
Q Is it your testimony, Doctor, that information from Wikipedia is a reliable resource when answering a scientific question?
A No, that is not my testimony. That is not my testimony, no.
Q Do you -- do you think you used Wikipedia here in writing your report?
A Again, I -- I -- I don't recall using Wikipedia specifically.
Q Okay. Let's take a look at your report at page 7, Doctor.

Page 72

And we'll mark a Wikipedia page as Exhibit 10.
(DEPOSITION EXHIBIT NUMBER 10 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q I would like to direct you, Dr. Levy, to the first full paragraph in your expert report at page 7.
A Uh-huh.
Q Do you see that?
A I do.
Q And I want to direct your attention to the sentence in the middle of that paragraph that begins "BRCA1 combined."
Do you see that?
A Yes.
MS. BROWN:
Q And I want to, side by side with Wikipedia, direct your attention to the third full paragraph that begins, as well, "BRCA1 combined."
You with me?
A I am.
Q Wikipedia writes, "BRCA1 combines with

Page 73

other tumor suppressors, DNA damage sensors, and single transducers to form a large multi-subunit protein complex known as BRCA1-associated genome surveillance complex" -- parens -- "BAC-" -- excuse me -- "(BASC)," end parens.
Do you see that?
A I do.
Q Turning to your report, page 7, you write, "BRCA1 combines with other tumor suppressors," comma, "DNA damage sensors, and signal transducers to form a large multi-subunit protein complex known as the BRCA1-associated genome surveillance complex" -- parens -- (BASC)."
Correct?
A That is correct.
Q Those two sentences, Doctor, are identical.
A It appears so, yes.
Q Okay.
A Except for a -- the reference included on the Wikipedia page is not included in my report.
Q Wikipedia has cited a reference, and

Page 74

your sentence stands without a reference. Is that right?
A That's right.
Q Other than the footnote, the two sentences we just read are identical. True?
A Both sentences state the same fact in the same way. So, similar to our earlier discussions, we've now seen a large collection of fundamental factual information with -- with accurate information from now a number of sources that are stated in similar ways through Wikipedia, other expert reports, and websites all about the fundamentals of cancer.
Q The two sentences we just read, Doctor, are identical. Correct?
MS. O'DELL:
Object to the form.
A We read one sentence in Wikipedia.
MS. BROWN:
Q And it is identical. True?
A Yes. The wording is the same. With, of course, Wikipedia, as you already stated, being editable by anybody and can pull that content from anywhere, and it's the -- I'd have

Page 75

to review -- I'd have to look to see what reference 16 in Wikipedia is. But it's certainly possible that I and Wikipedia summarized the same information from the same source.
Q Let's go to page 9 of your report. One of the articles that you relied on is an article by Lisa Coussens and Zena Werb. Do you recall that?
A That does sound familiar, but I'll have to verify.
Q Handing you what we've marked as Exhibit 12 [sic] to your report, the Coussens and Werb article.
(DEPOSITION EXHIBIT NUMBER 11 WAS MARKED FOR IDENTIFICATION.)
A Yes, this is a -- this is a review. This is an insight review article, which, similar to my report, is likely consolidating information from the research knowledge.
MS. BROWN:
Q I'd like to direct you to the last two sentences of Exhibit 10, the Coussens' article, the last two sentences in the first paragraph.
A Exhibit 10 or 12?

Page 76

Q I'm sorry. What did we mark the Coussens as? 12?
A Twelve.
Q That should have been 11.
We have marked the Coussens' article now correctly as Exhibit 11, and I'll direct you to the last two sentences of the first full paragraph. Put that, if you would, Doctor, side by side with your report at page 9, sentence that begins "in contrast," both sentences that begin "in contrast."
Are you with me?
A I am.
Q All right. So, in this published article, Ms. or Dr. Coussens writes, "In contrast, proliferating cells that sustain DNA" --
MS. O'DELL:
Excuse me, Alli. Sorry. Tell me, are you in the second paragraph?
MS. BROWN:
I'm on the end of the first full paragraph.
MS. O'DELL:

Page 77

Sorry. I thought you were in the first full paragraph.
MS. BROWN:
Begins "In contrast."
MS. O'DELL:
Okay.
MS. BROWN:
And we have that side by side with Dr. Levy's report, page 9, the paragraph that also begins "In contrast."
MS. O'DELL:
Thank you.
MS. BROWN:
Q Dr. Coussens writes, "In contrast, proliferating cells that sustain DNA damage and/or mutagenic assault" -- parens -- "(for example, initiated cells), continue to proliferate in microenvironments rich in inflammatory cells and growth/survival factors that support their growth."
Do you see that sentence?
A I do.
Q The next sentence reads, "In a sense," comma, "tumors act as wounds that fail to heal."

Page 78

See that?
A I do.
Q Directing your attention to page 9 of your report, Doctor, you write, "In contrast," comma, "proliferating cells that sustain DNA damage and/or mutagenic insult -- parens -- "(for example," comma, "initiated cells)," end paren, "continue to proliferate in microenvironments rich in inflammatory cells and growth/survival factors that support their growth," period. "In a sense, tumors act as wounds that fail to heal."
Do you see that?
A I do.
Q Except for one word, Doctor, those two sentences, including the slashes and the parentheses, are identical. Correct?
MS. O'DELL:
Object to the form.
A Those two sentences are similar.
MS. BROWN:
Q Except for one word, those two sentences are identical. True?
MS. O'DELL:
Object to the form. Asked and

Page 79

answered.
A Yeah. I'd certainly appreciate the similarity between the -- between the two. But that's -- again, as we've been discussing now for an extensive amount of time, in the introductory review content of the report --
In fact, I reference the Coussens and Werb paper, so certainly it's not a surprise that wording is similar between them and used similar language to describe, again, these factual aspects of fundamental cancer biology, including similar references.
MS. O'DELL:
Excuse me. My microphone is broken.
VIDEOGRAPHER:
It's still working. You're good. You can just lay it on the table and we'll fix it at a break.
MS. O'DELL:
And we've been going about an hour and 13 minutes.
MS. BROWN:
I'm about to finish up this section. We'll take a break.

Page 80

Q My question, Doctor, was: Except for one word, the two sentences we just read from Coussens are identical to the two sentences in your report. Is that correct?
MS. O'DELL:
Object to the form.
A So, I -- as -- as stated, the two sentences are similar.
MS. BROWN:
Q Except for one word, they are identical. Is that correct?
MS. O'DELL:
Object to the form. He's asked -- you've asked the question. He's answered your question.
A Again, the two sentences are similar.
MS. BROWN:
Q Do you understand "identical," what "identical" means?
A Yes. Exactly the same.
Q Okay. Except for one word, those two sentences are exactly the same in the Coussens article and your report. True?
MS. O'DELL:

Page 81

Object to the form. Asked and answered.
A And we're -- we're saying the same thing in different ways, which is that the two sentences are similar, stating factual information about fundamental cancer biology and in two similar review articles.
MS. BROWN:
Q And the only difference is one word. Correct?
A Two sentences are similar.
Q My question was: The only difference is one word. True?
A Let me review again to be sure that we would -- before answering.
Taken out of context, those two sentences are similar.
Q My question was, Doctor, the only difference is one word. Is that correct?
MS. O'DELL:
Objection to the form. Asked and answered.
A You know, I think we've -- we've answered this a number of times, that the two

Page 82

sentences are different in their context and in terms of paragraph, but they are similar in structure and similar in wording.

But, as you stated, with the exception of the -- so they're not. So in a language perspective, they're not identical. They're similar.

MS. BROWN:

Let's take a break.

VIDEOGRAPHER:

Going off -- going off the record. The time is 10:15 a m.

(OFF THE RECORD.)

VIDEOGRAPHER:

We're back on the record. The time is 10:25 a m.

MS. BROWN:

Q Doctor, I am handing you what I have marked as Deposition Exhibit 12 and 13. These are additional documents your counsel identified for us this morning as something you have seen since your report.

(DEPOSITION EXHIBITS 12 AND 13 WERE MARKED FOR IDENTIFICATION.)

Page 83

MS. BROWN:

Q Would you tell us what those two exhibits are, please.

A Exhibit -- Exhibit 13 is a printed copy of an email dated December 26th informing Dr. Saed that a manuscript --

Is it helpful to identify the manuscript?

-- titled "Molecular Basis Supporting the Association of Talcum Powder Use With Increased Risk of Ovarian Cancer," submitted to Reproductive Sciences, has been reviewed. The comments were included in the letter.

Q Have you seen --

A And I'm just reading the --

Q Sure.

A It -- it appears that the -- so, summarizing the letter, the manuscript has been reviewed, the comments from the reviewers were provided back, and the journal has informed Dr. Saed that they'll accept a revised document for potential publication.

Q Have you seen Exhibit 13 prior to this morning?

Page 84

A I have.

Q Have you seen the reviewer comments referenced in Exhibit 13?

A I have not seen the reviewer comments.

Q Okay. Exhibit 13 does not inform the opinions of your report dated November of 2018. True?

A Exhibit 13, being the letter, that is correct. It does not.

Q Okay. And what's Exhibit 12?

A Exhibit 12 appears to be a preprint version of the previously mentioned paper, "Molecular Basis Supporting the Association of Talcum Powder Use With Increased Risk of Ovarian Cancer," with the first author, Nicole Fletcher, and Dr. Saed is listed as the senior or corresponding author.

Q Did the lawyers provide you with this manuscript, Doctor?

A Yes, in a -- but that's -- yes, they did.

Q Do you recall when you were provided with a copy of the manuscript by the plaintiffs' lawyers?

Page 85

A It was sometime in December toward -- late in the year. The exact date, I'd have to review when it came in. And I believe it was -- and the version you have here is a more formal preprint version from the -- from Manuscript Central, whereas the version I received was a -- it appeared to be more of a submission version.

So commenting whether it's exact -- precisely the same content, I -- I wouldn't be able to say.

Q Fair to say, though, Doctor, since you received the manuscript in December of 2018, the contents of the manuscript did not inform the expert report that you wrote in November of 2018; correct?

A Actually, I would say the -- the -- I would not agree, from the perspective of Dr. Saed has a number of similar studies, as well as a number of abstracts that I had the opportunity to review that did inform some of the opinions in the report. Those same information and data were included in this manuscript and expanded upon actually significantly.

Page 86

So the basis of my opinion includes some of the information from this manuscript, but I -- but the report does not contain the totality of this.
Q Right. Because the manuscript wasn't available to you until after you wrote your report. Right?
A No, that's not the case. The -- the -- the research, some of the research information from this study was available in abstract form, and -- and some -- I believe a preprint from Dr. Saed.
So it was -- so it was available. Portions of it were available for the report.
Q Other than the abstract, did you have access to an earlier version of what we've marked as Exhibit 12?
A I can't accurately answer that without comparing them.
Q Where do you have stored the earlier version that you're referring to?
A Let's see if I -- what I have here.
So, from Dr. Saed, I have a -- used a book chapter which describes some of his

Page 87

oxidative stress experiments that are also consistent with the information that's in the -- in Exhibit 12, as well as some of his earlier review articles, and that's --
Let me make sure I'm not missing anything from Fletcher, who's been...
But, otherwise, the -- the experiments that were expanded upon in the formal manuscript were described in -- in abstract or, I should say, summarized form, meaning an abstract that included methods, results, and conclusions from Fletcher and colleagues in Dr. Saed's group.
Q At the time you wrote your report, you had an abstract of the 2018 paper that we've marked as Exhibit 12; correct?
MS. O'DELL:
Object to the form. He said plural.
A Yes. I had two abstracts and then possibly --
I'd have to review when I received this preprint versus the final version of my report to see if they overlapped, if they're -- if I had an opportunity to review this or not.
MS. BROWN:

Page 88

Q Okay. And I'll ask if you'd be kind enough to do that at a break. Just let us know if you had access to something other than the abstract of Dr. Saed's 2018 report at the time you wrote your report. Fair enough?
A I'll make a note.
MS. O'DELL:
Excuse me. Object to the form. Abstracts, not one.
MS. BROWN:
Q Dr. Levy, you are a Ph.D.; is that correct?
A Correct.
Q Okay. You are not an M.D.; correct?
A That's correct.
Q What's your Ph.D. in, sir?
A Biochemistry and genetics.
Q You're not an epidemiologist. Fair?
A I am not.
Q Okay. And the focus of your work at HudsonAlpha is on genome sequencing. Is that right?
A No. The -- the -- genome sequencing is a tool that we apply in -- in the work of my

Page 89

laboratory and in my responsibilities at HudsonAlpha.
Q HudsonAlpha has a team known as the Breakthrough Breast and Ovarian Cancer Team. Is that right?
A I'm not familiar with that name.
Q Okay.
A There is a -- a group of faculty who have some funding related to breast and ovarian cancer. It's -- it's certainly possible that name was used in -- in press for some title.
Q Since you're not familiar with that team, fair to say you're not a member of the Breakthrough Breast and Ovarian Cancer Team?
MS. O'DELL:
Object to the form.
A Again, I don't -- my involvement with breast and ovarian cancer at HudsonAlpha is specific to some projects. And whether or not I was named on that team, I -- I don't know.
MS. BROWN:
Q There are folks at HudsonAlpha, scientists and doctors at HudsonAlpha whose practice is devoted to studying ovarian cancer.

Page 90

Correct?
A No, that's not correct.
Q Your practice is not devoted to ovarian cancer; correct?
MS. O'DELL:
Object to the form.
A No. My -- my practice is not devoted to ovarian cancer. And -- but that was irrelevant to what I was asked to do in this -- in this particular case for the -- regarding the content of my report.
MS. BROWN:
Q I think I saw you've published one article regarding ovarian cancer over the course of your career. Is that right?
A That sounds correct.
Q You have not given any presentations regarding ovarian cancer. Is that true?
A I would say that's accurate.
Q You have not received any government funding to study ovarian cancer. True?
A I received government funding to study breast and ovarian cancer -- this was in 2002, from the Department of Defense -- and then,

Page 91

subsequent to that, participated in at least one review for the Department of Defense in reviewing ovarian cancer research grants. So that is --
And then my membership in the Vanderbilt Cancer Center as well as the University of Alabama Birmingham Comprehensive Cancer Center certainly have been involved in a number of projects across a diversity of cancer types, including ovarian and breast cancer.
Q Prior to being hired by the plaintiffs' lawyers in this litigation, you had not investigated the potential mechanisms by which talcum powder could cause ovarian cancer. Is that fair?
MS. O'DELL:
Object to the form.
A Specific -- as in terms of a specific fundamental research project?
MS. BROWN:
Q At all.
MS. O'DELL:
Object to the form.
A So my research has included the role of inflammation and a number of biological processes

Page 92

dating back to my early Ph.D. work, and those include cancer. So certainly the subject of inflammatory response in -- both chronic and acute, in controlling cancer has been a subject of my research for some time and certainly bridged into ovarian cancer as well as other cancer types.
MS. BROWN:
Q You've never published on chronic inflammation as a potential mechanism by which talcum powder causes ovarian cancer. Correct?
A Not specific to talcum powder, no.
Q You have never given a presentation on chronic inflammation as a mechanism for causing ovarian cancer at all; right?
MS. O'DELL:
Object to the form.
A I'm thinking through my --
I don't recall a specific presentation with regards to talcum powder and its role in ovarian cancer. As far as my discussions or presentations around the role of inflammation in cancer, including ovarian, it -- it is -- it is possible, but I can't think of a specific

Page 93

presentation.
MS. BROWN:
Q Okay. Since you've been hired by plaintiffs' lawyers, you have done some research into the potential role of inflammation and ovarian cancer. Is that right?
MS. O'DELL:
Object to the form.
A Since -- since my -- what was requested of me from the plaintiffs' attorneys was to provide a review of the biological plausibility and a connection between talcum powder and inflammation and then discuss the relationship between inflammation and cancer.
MS. BROWN:
Q Okay. As I understand you, Dr. Levy, you were asked by the plaintiffs' lawyers to provide a review of the literature as it relates to the biological plausibility of talcum powder and ovarian cancer. Is that right?
MS. O'DELL:
Object to the form.
A No, that's not correct. What I was --
I was asked to provide an opin- -- expert opinion

Page 94

on the biological plausibility of the mechanism that -- of the ability of exposure of talc and its constituent components to cause inflammation and/or cancer.
MS. BROWN:
Q Do you see those as two different things?
A Yes.
Q Okay. So you were asked to provide a mechanism by which talcum powder could cause cancer?
A No, that's not correct.
MS. O'DELL:
Objection to form.
MS. BROWN:
Q Okay. Explain it to me.
A I -- I was asked to provide a -- an opinion on the biological plausibility --
Q Of talcum powder causing cancer?
A -- of talcum powder leading to the biological changes necessary to cause cancer.
Q Okay. As I understand what you just said, you were asked to re- -- to provide an opinion on the biological plausibility of talcum

Page 95

powder leading to biologic changes that are needed to cause cancer. Is that fair?
MS. O'DELL:
Object to the form.
A So I was asked from -- by the attorneys to review the available literature across the spectrum of cancer and talcum powder and constituent literature to develop an opinion around the biological plausibility that exposure of -- exposure to talcum powder is biologically -- that there is a biologically plausible mechanism that that can cause cancer.
MS. BROWN:
Q Okay. And that is not something that you had done prior to being hired by the plaintiffs' lawyers. Fair?
A Developing such an opinion?
Q Correct.
A Or -- or -- so writing such a report, no, that is not something I -- I had done prior to -- to this. My research has been primarily in data integration and the examination of mechanistic effects in cancer, rare disease, and -- and in diabetes specifically, as well as

Page 96

some neurological diseases.
So this was a similar review as -- of those topics when asked to examine the biological plausibility of a cause and effect; in this case, cause being exposure to talcum powder and effect being progression to cancer.
Q Prior to being hired by the plaintiffs' lawyers, you had not considered the biological plausibility of talcum powder causing ovarian cancer. Correct?
A No. I would say that's not true in isolation. And the reason I say that's not true is I had been aware of some of the literature and certainly some of the press that surrounded the suspected associations between talcum powder exposure and cancer. So I was familiar with the concept, but I had not at the time, until hired by the plaintiffs' attorney, spent a significant amount of time reviewing the literature and developing a written opinion as to that biological plausibility.
Q You have not published your opinion contained in -- your opinions contained in the report that we marked as Exhibit 2. Is that

Page 97

correct?
A That is correct.
Q You have not presented the opinions contained in Exhibit 2 at any medical or scientific conference; correct?
A That's correct.
Q You have not disclosed the opinions contained in Exhibit 2 to any of your colleagues; correct?
MS. O'DELL:
Object to the form.
A Not at this time, no. Considering I had -- I had just finalized the report a short time ago, I haven't had the opportunity to consider publication, presentation, or -- or discussion with colleagues.
MS. BROWN:
Q Do you plan to seek publication of the information contained in your report in Exhibit 2?
A I -- I haven't made a determination at this time. It's been a fascinating area to research. Certainly there's -- that would certainly be a future possibility.

Page 98

Q Does HudsonAlpha --
First of all, what's your position at HudsonAlpha, Doctor?
A So I'm a faculty investigator, which would be analogous to a faculty member at a research institution, similar to -- or I should take a step back and just --
To be accurate, HudsonAlpha is a private nonprofit research institution, similar to Broad Institute, Stowers, et cetera. So we are academic in nature, meaning that most of our funding or the vast majority of our funding comes from grants and contracts. So that's why I say it's analogous to faculty at a research institution.
My other responsibilities are the management and oversight of the production and research laboratories, so that provides us an opportunity to work with approximately 1200 different laboratories from around the world in support of roughly 5,000 projects over the last nine and a half years. And that's -- it's provided a broad spectrum of activities and abilities to work in these types of projects.

Page 99

And then I also oversee the clinical laboratories as well. And adult oncology is a major focus of that research. I currently lead the largest profiling effort in adult cancer in the nation, which involves 15 national cancer institutes. And ovarian cancer is a component of that research, although not the only cancer that we research in that -- in that's -- in that program.
Q None of the 5,000 projects you just mentioned have dealt with talc. Is that fair?
A That is fair.
Q And none of the work at the clinical labs that you just mentioned have dealt with talc; correct?
MS. O'DELL:
Object.
A I am -- I would say there's a statistical probability that some of the ovarian cancer samples that have been observed in the clinical laboratory may very well have been -- have come from patients exposed to talcum powder. But I have no direct knowledge of that, nor have we performed any testing to confirm

Page 100

or -- or -- or dispute whether or not those ovarian cancer or other cancer types may have had a relationship to talcum powder. So the short answer being I -- I don't have the information to answer that.
MS. BROWN:
Q HudsonAlpha has a Code of Ethics. Are you familiar with it?
A Yes.
Q Are you familiar with the financial disclosure requirements of HudsonAlpha?
A I am.
Q Have you complied with those in connection with your work as an expert witness for plaintiffs in this case?
A I have.
Q And tell us what you've done to comply with HudsonAlpha's Code of Ethics and financial disclosure requirements.
A Their Code of Ethics and financial requirement is requirement to disclose any relationships that have a financial component over -- I don't recall the minimum amount, but it is -- it is fairly modest, hundreds of dollars.

Page 101

And that reporting requirement is the -- is -- is for the previous year, and it is due in July, I believe is the time frame, although I'd have to make sure. It's -- I know it's not the end of the calendar year. So on my next disclosure, this, of course, activity would be disclosed.
In addition to that, via conversation -- regular review with the president of the institution, I provide a general report on consulting activities; for example, these activities.
HudsonAlpha's policy is faculty members are allowed up to 20 percent of your time towards consulting activities that have a relationship to your research area, such as the evaluation of the biologically plausible mechanism of talc in ovarian cancer. So based on both the timing of the Code of Ethics with regards to the financial disclosure as well as the ad hoc reporting of consulting engagements with the president of the institution, I'm in compliance with the current policies of HudsonAlpha.
Q The president of HudsonAlpha is aware of your opinions in this case?

Page 102

A I have not discussed my opinions specifically to this case with him; just the general knowledge that I was asked to participate as an expert witness. He didn't ask, and I didn't provide the content.
Q No one at HudsonAlpha is aware of your opinion that talcum powder causes chronic inflammation which can cause ovarian cancer? Is that right?
A I have -- I have not specifically shared the contents of the report or -- or my opinions widely at HudsonAlpha.
Q Did you disclose last July that you had already been hired and submitted invoices to the plaintiffs' lawyers?
A I'm sure I did.
Q Do you have that documentation?
A No. It's -- it's an electronic disclosure. It's not actually done on paper.
Q One of the things that HudsonAlpha does is it partners with the University of Alabama in a comprehensive cancer center; correct?
A No, that wouldn't be correct. HudsonAlpha is very specific --

Page 103

And you may be more familiar with this than I.
They're very specific with their use of the word "partnership" and they're, in fact, very specific that they do not engage in a -- anything titled "a partnership." So they -- I would not characterize them as a partner of the University of Alabama Cancer Center.
We certainly have -- there are faculty members at University of Alabama Birmingham who are -- have adjunct appointments at HudsonAlpha, just as I have appointments at University of Alabama Birmingham and I am a member of their cancer center.
Q Are you aware of the work that HudsonAlpha does with the University of Alabama's Comprehensive Cancer Center?
MS. O'DELL:
Object to the form. Asked and answered.
A I'm aware of some of the work, but I -- certainly I -- I don't -- I don't necessarily have knowledge of the full spectrum of those projects, given that they involve many faculty

Page 104

members on both institutions.
MS. BROWN:
Q Fair to say, then, Doctor, you have not participated in any work with the University of Alabama's Comprehensive Cancer Center?
MS. O'DELL:
Object to the form.
A No, that's not true.
MS. BROWN:
Q Have you worked with the University of Alabama's Comprehensive Cancer Center on projects involving ovarian cancer?
MS. O'DELL:
Objection. Asked and answered.
A I would -- I would have to review the specific projects that we've -- we've done to answer that.
As the codirector of a core facility for the University of Alabama Comprehensive Cancer Center, it is likely that we've worked on some projects related to ovarian cancer, but I can't specifically name them. They are -- I would -- I would characterize them as infrequent.
MS. BROWN:

Page 105

Q Have any of those projects attempted to research the potential causes of ovarian cancer?
A Again, I'd have -- I'd have to review the projects. They're certainly -- fundamentally, most of the questions regarding the analysis of cancer samples are routinely to investigate their cause or their treatment. So I would -- I would answer that question as highly likely.
Q Would you agree the cause of ovarian cancer remains unknown today?
MS. O'DELL:
Object to the form.
A No, I would -- I would -- I would not agree that it -- I would not agree to that general statement.
MS. BROWN:
Q What are the causes of ovarian cancer in your mind, Doctor?
A Well, the -- the causes of -- of a -- of any number of cancers, including ovarian cancer, are probably more well understood now than ever, and their complexities I think now are just beginning to be appreciated in the sense

Page 106

that cancer is a disease of unregulated cell growth.

Back to our earlier con- -- earlier conversation, some of the fundamental facts that we had discussed and, in fact, I think well replicated in a number of sources, as you pointed out to me, you know, illustrate that there's a milieu of genetic change leading to cellular transformation, and that cellular damage, if we consolidate that as cellular damage, then has to work in concert with a number of other events providing the right environment for a tumor to grow, such as inflammation, chronic or acute. And, so, the -- you know, the -- the -- you know, giving a singular cause would be inappropriate.

But I would say the mechanistic causes of cancer are reasonably well understood, but how those apply to the wide diversity of cancer types remains an area of active investigation.

I think what's interesting on cancer in general is that there's no -- really no longer a bucket diagnosis. It is -- it -- lung cancer is more complex than lung cancer and ovarian cancer, certainly with the --

Page 107

As I'm sure you're well aware, with the molecular subtypes and other things, it's a complicated disease as well.

So to summarize that is -- to summarize all of that complexity by saying that the cause is known or unknown I think would vastly underestimate the -- our current state of the art or knowledge of how complex cancer is as a condition.

Q Sure.

Scientists, researchers, public health authorities continue to investigate the mechanism by which ovarian cancer is caused. Correct?

A That's correct.

Q We do not, sitting here today in 2019, have a complete understanding of the etiology of ovarian cancer. Correct?

MS. O'DELL:

Object to the form.

A I would say we have substantial knowledge of factors and exposures that either predispose or directly cause cancer in a large number of -- large number of cancer areas, including ovarian cancer.

Page 108

Now, the -- whether that represents the complete milieu of possibilities is -- is what is currently under research.

MS. BROWN:

Q Were you aware that the University of Alabama Comprehensive Cancer Center is an NCI center, National Cancer Institute?

A Yes. It's -- it's not only an NCI-designated center; it's an NCI-designated comprehensive cancer center, which is a slightly different classification. It's a -- there's more criteria for a cancer center to meet to become comprehensive.

Q What does it mean to be an NCI center, to you, if you know?

A Stated very simply, it means you have a -- your cancer center is funded by a support grant directly from the National Cancer Institute to provide -- that supports not only patient care but also supports basic research, epidemiology and -- and health outcomes research in cancer.

So, in a nutshell, it is a fairly comprehensive grant that supports a wide variety of work within a cancer center that extends

Page 109

beyond basic -- basic care.

Q The National Cancer Institute has funded a number of projects that the scientists at HudsonAlpha are working on. Is that fair?

A I'd have to certainly review the grant portfolio. But I'm certain that, since I myself have funding from that cancer center, yes, the NCI does fund some -- some number of investigators at HudsonAlpha.

Q And you consider the NCI to be a reputable public health authority; correct?

A No, not necessarily. The NCI is really not a public health authority. The N -- the NCI is a -- is a scientific administration center within the National Institutes of Health.

Now, I'm speaking of their extramural programs. The NCI also have intramural programs, where they have their own researchers and their own projects. I'm less familiar with those activities.

But together, I would state that the NCI is a -- I don't have -- I guess I have not had any experience with the NCI that would lead me to say that they are an authoritative public

Page 110

health authority.
Q Before forming your opinions in this case, Dr. Levy, did you look to see what the NCI states about whether talcum powder causes ovarian cancer?
A I believe I did see, from a number of statements, certainly potentially from the NCI, regarding the complete opinion and -- and knowledge base for the role of talcum powder in ovarian cancer.
Q Do you recall that the NCI has concluded that there's inadequate evidence that talcum powder increases the risk of ovarian cancer?
MS. O'DELL:
Object to the form.
A Which -- what specifically are you referring to? I -- I wouldn't be able to answer that accurately without knowing which specific report or statement that you're referring to.
MS. BROWN:
Q I'm wondering if, sitting here today, you recall looking at information about the classification of risk factors for ovarian cancer

Page 111

as done by the NCI.
A I don't recall that specifically. I don't also recall seeing any statements from the NCI regarding safety of any product.
Q In forming your opinions in this case, Dr. Levy, did you consider the conclusions of public health authorities like the FDA, the NCI, NIH as it relates to talcum powder in ovarian cancer?
A So I certainly considered information from each of those entities. But I would make a statement I don't -- I don't recall from any of those entities seeing a single conclusion.
Q Is it your opinion, Dr. Levy, that talcum powder causes ovarian cancer?
A I wasn't asked to provide an opinion if talcum powder causes cancer. I was -- I was asked to develop an opinion as to the biological plausibility of -- of talcum powder leading to -- leading to change.
Now, that's what I was asked from the attorneys. If you're asking -- are you asking me what my opinion is --
Q Well, I want to know if, in this case,

Page 112

you are prepared to offer the opinion that talcum powder causes ovarian cancer.
A I don't -- I don't think we have the complete information for a sing- -- you know, to have the opinion of a singular cause. But, at the same time, my opinions are that, as stated in the report, there's a clear and well-evidenced biologically plausible role for talcum powder leading to ovarian cancer.
Q On page 2 of your report, the second full paragraph that begins "My report consists" --
You with me?
A Yes.
Q -- you state -- you reference your conclusions regarding this cause-and-effect relationship.
Do you see that?
A I do.
Q Do you mean by that that you have an opinion that talcum powder causes the effect of ovarian cancer?
A No. That -- that wasn't the meaning of that statement of cause and effect. It was -- it

Page 113

was a -- more of a general statement of a cause being exposure to talc and effect being that biologically plausible mechanism.
Q You mentioned a moment ago that you don't think we have the complete info on a singular cause of ovarian cancer. Is that right?
MS. O'DELL:
Objection to form.
A Sorry. Let me read your question again.
I have -- I have not seen any evidence that suggests that there is a singular cause of ovarian cancer.
MS. BROWN:
Q You have not seen sufficient evidence to suggest that talcum powder could be one of the causes of ovarian cancer; correct?
MS. O'DELL:
Object to the form.
A I would disagree. As -- as stated, the -- I have not seen evidence that there's a singular cause of ovarian cancer. I think there is ample evidence that there are a multitude of mechanisms that you can get cellular damage and

Page 114

cellular change within the ovary which then leads to malignant transformation, and that, as stated in the report, there's a biologically plausible mechanism that exposure to talcum powder and its constituents can create those necessary changes.
MS. BROWN:
Q Do you believe, Doctor, there's sufficient evidence that talcum powder, through chronic inflammation, causes ovarian cancer in some individuals?
A No. That -- that was not my -- not my opinion or statement. And I would say specifically chronic inflammation is, again, narrowing the focus in an inappropriate way, and the evidence doesn't illustrate that chronic inflammation is a singular sufficient detail or, I should say, effect to result in ovarian cancer. It's certainly a factor, as -- as well described in the -- in the literature.
And -- and, again, I would defer to other expert reports that have similar opinions regarding inflammation, chronic inflammation being one of them.
And it may be important to provide an

Page 115

important distinction that cellular damage or what we can refer to as acute inflammation can cause -- certainly has been shown and is well-evidenced that it causes -- can lead to molecular changes that can lead to cancer.
Chronic inflammation is a slightly -- is in a slightly different biological perspective in that it provides the correct environment for those cancerous changes to take hold and allow malignant transformation, as I mentioned.
So I -- I do view them as working in concert but not necessarily independent. So when you ask a question that specifically narrows it to chronic inflammation or even acute inflammation in a singular fashion, you know, my answers will largely be the same, that that's, in and of itself, is too limited to describe as a specific cause, singular or otherwise, of ovarian cancer or of cancer in general.
Q You'd agree that the research regarding whether chronic inflammation can cause ovarian cancer is ongoing?
A Yes, I would agree it is -- it is ongoing research. But there are a large number

Page 116

of observations and studies that have -- certainly exist. And, again, their review and -- and content is what went to the opinions in my report.
Q And most of the studies that you cite, Dr. Levy, talking about chronic inflammation refer to chronic inflammation as a hypothesis of one of the ways cancer might form in the ovary. Correct?
MS. O'DELL:
Object to the form.
A Let me -- sorry. Let me read your question.
No. I would disagree. At least, certainly not most of the studies that I cite.
MS. BROWN:
Q Do you believe chronic inflammation is an established mechanism of ovarian cancer?
A Yes, in the sense that chronic inflammation is a well-established mechanism of cancer in general, including ovarian cancer. This is first observed in the 1800s and has since been -- become well-established in the -- in the cancer field that inflammation plays a

Page 117

significant role in both the initiation as well as progression of cancer.
Q What methodology did you employ for coming to the opinion that chronic inflammation is a well-established cause of ovarian cancer?
A Just general mechanism in terms of evaluating biological plausibility.
Q I understand, Dr. Levy, you have a general opinion that chronic inflammation can lead to some cancer. Is that right?
MS. O'DELL:
Objection to form. Misstates his testimony.
A I -- I have an opinion regarding the role and importance of inflammation in the initiation and progression of cancer.
MS. BROWN:
Q And, as it relates to ovarian cancer, what methodology did you employ to arrive at your conclusion that chronic inflammation is an established cause of ovarian cancer?
A I -- I did not arrive at that specific conclusion, nor was I asked to.
Q You do not believe that chronic

Page 118

inflammation has been established as a cause of ovarian cancer; correct?
MS. O'DELL:
Object to the form.
A No, that -- that's not what I said.
MS. BROWN:
Q Explain it to me.
A I've stated that chronic inflammation or inflammation in general, including chronic and acute infor -- inflammation, is a component and a necessary component for the initiation and progression of -- of cancer as we understand it today. And, in that, cancer, certainly ovarian cancer as well as a variety of other cancer types, is included.
Q What methodology did you employ to arrive at the conclusion that ovarian cancer is one of the cancers that can be caused by chronic inflammation?
MS. O'DELL:
Object to the form. Misstates his testimony.
A Yeah. Again, we're not -- I'm not making a specific causal opinion with respect to

Page 119

any -- whether -- whether inflammation, talcum powder use or other exposures. I -- my -- my opinion in the report is -- is -- was not asked to be a causal opinion.
MS. BROWN:
Q You reference on page 2 of your report that your opinions are based on assessing and weighing the totality of the evidence, including relevant literature and available documentation and your experience as a geneticist and scientific researcher. Do you see that?
A Yes.
Q What do you mean by "the totality of the evidence"?
A All of the evidence available at the time that I was researching this report.
Q All of the evidence concerning what?
A Concerning a variety of subjects surrounding ovarian cancer, talcum powder use, and then inflammation and related subjects as my literature review and review of available information progressed.
So there was a, I guess, a large number of tangential directions that -- that I examined,

Page 120

from animal models to in vitro studies, in vivo studies, cohort studies, case-control studies. There was quite a broad spectrum of information across a large number of years.
Q Do you believe you reviewed the totality of the epidemiology on talcum powder use and ovarian cancer?
MS. O'DELL:
Object to the form.
A I -- I reviewed the available studies that appeared to be relevant for the -- for the opinions that are expressed in my report.
MS. BROWN:
Q And when you say "available," what do you mean?
A Meaning that I could -- I could discover in the scientific literature.
Q Did you conduct your own literature searches in connection with your work in this case?
A I did.
Q How did you go about finding the totality of the evidence relating to whether talcum powder causes ovarian cancer?

Page 121

A So the -- my methodology for the literature review in establishing my opinion regarding the biological plausibility of talcum powder exposure inflammation and its potential role in ovarian cancer was based on, you know, my activities and many other literature searches, so using a variety of computational tools and -- and web-based resources, from journals to, I would say, primarily PubMed being a resource, but also ISI, Web of Science, Google Scholar and a variety of -- bioRxiv and I'm sure a number of other sources. But those were probably the more primary resources for establishing what literature was available.
Q Did you ask the plaintiffs' lawyers for any scientific literature that you used in forming your opinions in this case?
A What do you mean by "ask"? There is -- as far as did I ask for their similar process, no.
There were some papers that I had identified but was not able to access the full content via the libraries that I have access to. So in some of those cases, specific references

Page 122

that I provided, those full -- that full content was provided by the plaintiffs' lawyer to allow me to review it.
Q Did the plaintiffs' lawyers give you a set of epidemiology on which you're relying on to form your opinion?
A No, they did not.
Q If I look at your report, I see a reference list and then a separate Exhibit B. Is that right?
A Yes.
Q So, for example, on page 18 of your report, you have a list of literature cited. Correct?
A Yes.
Let me make sure I have the page correct.
Yes, beginning on page 18.
Q Is everything that appears in the literature-cited list something that you found on your own, Dr. Levy?
A I would have to review the -- the list. But there are certainly --
Let me --

Page 123

I believe the Saed abstracts, as an example --
Let me see if there are --
No. I -- I believe, in the literature cited, there are certainly some number of examples of information that was provided during the course of the development of my report from the plaintiffs' attorneys in terms of literature for my consideration, but that in no case -- in every case it was provided as a -- as information.
The vast majority or nearly the totality of this was information that I had -- that I indeed discovered myself and shared with the -- the attorneys, but certainly not complete.
Q On page 18 you cite an article by Blount.
Do you see that?
A Yes.
Q Was that given to you by the plaintiffs' lawyers?
A I'd have to look at my records. I don't recall.
Q Off of the top of your head, are you

Page 124

relying on information in that article to form your opinions in this case?
A No. I'm not relying on any singular article or source to form my opinion on the case.
Q Are you relying in part on the information contained in the Blount article?
A Since I include it in the cited literature, certainly in some -- in some part.
Q What information are you relying on in the Blount article?
A I would have to review the article to remind myself where the --
Q Take a look at it. We'll pull it right now.
What about Paoletti on page 22? Was that something you found on your own or did the lawyers give you that?
A So Paoletti --
Q Uh-huh.
A Page 22?
Q Uh-huh.
A Actually, the Paoletti one is familiar. That's an interesting one because it's in Italian.

Page 125

Q Are you relying on the information in the Paoletti article to form your opinions in the case?
A Again, the -- I wasn't relying on any singular article but instead tried to present and provide reference to as comprehensive a collection of relevant literature in this -- in this space as possible, of which Paoletti, although being in Italian, there were some -- enough translated aspects of that that it was worthy to include in the -- in that cited literature as being relevant to the -- to those -- to those opinions.
Q Just to make sure we get on the same page here, Dr. Levy, when I ask are you relying on something, I don't mean by that question to suggest it's the only thing you're relying on. And I'll try to say "in part" to make it easy for us. Okay?
A Right. Just want to be -- make sure we're clear.
Q Absolutely. So do I.
And I want to know are you relying in part on anything in the Paoletti article to form

Page 126

your opinions in this case?
A I would say in -- in part. As far as my opinions regarding the biologically plausible mechanism that was presented, no, it does not rely on that specific conclusions of that paper but, rather, that paper was included because of its results regarding asbestos contamination in industrial talc, which only support -- add support to the mechanism that I presented in the report.
Q Is your opinion in this case, Doctor, based on an assumption that baby powder contains asbestos?
A No, it is not.
MS. O'DELL:
Object to the form.
MS. BROWN:
Q Is your opinion in this case based on an assumption that baby powder contains fragrances?
MS. O'DELL:
Objection to form.
A My -- my opinion considers the totality of the constituent components of baby powder,

Page 127

Shower to Shower, you know, under -- either, as we've been referring to it simply as talc or talcum powder or by trade names such as Johnson & Johnson or Shower to Shower, so the -- my opinions, as stated in the report, being reasonably -- or trying to be reasonably comprehensive. Therefore, it's not, you know, limited to any -- any singular component, whether it be majority or minority, in the -- in the talcum powder products, as I just stated.
MS. BROWN:
Q Is your opinion in this case based on an assumption that Johnson & Johnson baby powder products contain heavy metals?
MS. O'DELL:
Objection to form.
A Again, similar to the earlier statement, the opinion is not subject to any -- any singular component. I think the information regarding the -- in deferring to some of the other experts regarding the knowledge of constituent components, whether they be heavy metals or asbestos, only helps to support the biological plausibility of the mechanism I

Page 128

presented.
MS. BROWN:
Q Do you believe that baby talc alone can cause inflammation that may lead to ovarian cancer?
A Based on my review of the literature, there are a number of studies, both of those involving human studies in terms of case controls, as well as a number of animal studies and then, more specifically, in vitro studies that look at talcum powder and its ability to produce clear markers of inflammation.
I am -- the -- I am not aware of any specific testing that looked at platy talc individually as a singular component without the -- or out of the context of the products we were just describing in a similar analysis. So I don't -- I don't know that answer.
Q Is it your opinion that Johnson & Johnson baby powder products are contaminated with asbestos?
MS. O'DELL:
Object to the form. Asked and answered.

Page 129

A I -- I -- I have -- I have been provided expert report, and some of those are referenced in the -- in the report, as we were describing, that describe testing of a number of -- number of samples, included -- Johnson & Johnson included in that, that showed how they -- that the results of those reports showed contamination by asbestos or -- or -- or asbestos-like fiber. So, therefore, I've been presented with that evidence.
MS. BROWN:
Q Have you relied on that evidence in forming your opinions in this case?
A Again, no, not -- not as a singular evidence. So, as we just discussed a moment ago, that is a component piece of evidence that leads -- and is supportive of the biologically plausible mechanism described in the report.
You know, certainly, it is inarguable that asbestos and asbestos-like fibers cause inflammation. There's also ample evidence of the inflammatory effects of talc. And -- and talc pleurodesis, for example, is -- is designed to produce inflammatory response as a treatment.

Page 130

So I think, again, similar to the relationship of asbestos and inflammation, it's a well-established scientific fact that talc has an inflammatory role now. Or I should say as of today.
Q Have you attempted to quantify, based on the reports of Dr. Longo that you reviewed, how much asbestos contamination is in Johnson & Johnson baby powder products?
MS. O'DELL:
Objection. Vague as to form.
A I --
MS. O'DELL:
As to the volume and time contained, et cetera.
A My -- my answer is simply that I wasn't asked to quantify that as part of my report.
MS. BROWN:
Q Whether there is asbestos in Johnson & Johnson baby powder products or not does not impact your opinions in this case; is that right?
MS. O'DELL:
Object to the form.
A The opinions regarding the biological

Page 131

plausibility described in my report and its relationship to asbestos are somewhat separate, meaning that I have -- I was not able to discover what the contamination rate or content of asbestos was in any of the referenced studies through the course of my report, so, therefore, I can't comment on the likelihood or -- of -- of how many or any -- or any or all of those samples contain asbestos.
MS. BROWN:
Q And sounds like you did some work attempting to see if you could calculate a contamination rate. Is that what you were describing?
MS. O'DELL:
Object -- object to the form. Misstates his testimony.
A No. No, not at all. I stated that I didn't have information available to assess either -- either way.
MS. BROWN:
Q Tell me what you meant when you testified that you were not able to discover what the contamination rate or content of asbestos was

Page 132

in any of the above-referenced studies.
MS. O'DELL:
Objection. Misstates his testimony.
A So reading -- reading back my testimony --
MS. BROWN:
Q So, Doctor, I see that you're looking at the realtime?
A Yes.
Q To get clarification on the question?
A No. To -- to remem- -- to -- you asked me a question about my statement.
Q Correct.
A And I was reviewing specifically what I had stated so I could answer your question accurately.
Q Terrific. So I want to know what you were talking about when you said you were unable to discover the contamination rate.
A To clarify, I was not asked to estimate or determine the contamination rate, and my statement regarding that was in reference to the material I reviewed and the literature that is referenced in my report. I don't recall in any

Page 133

of those studies observing a specific statement of amount of asbestos in the talcum powder products that were under study. So, therefore, I am not able to form an opinion surrounding that contamination rate.
Q Would the same be true, Doctor, for heavy metals?
A Yes, that's correct.
Q And when I say the same would be true, that means you were not able to calculate a rate of heavy metal contamination of any of the talcum powder products in the studies you reviewed?
MS. O'DELL:
Objection. Vague.
A I was not asked to.
MS. BROWN:
Q Did you attempt to quantify the amount of heavy metals?
MS. O'DELL:
Objection.
A I certainly reviewed the literature to understand what information was available regarding the products that may have been used and what testing may have been done on

Page 134

those -- on those products.
MS. BROWN:
Q And, as it relates to fragrances, have you calculated the amount of fragrances that are present in Johnson & Johnson's baby powder products?
MS. O'DELL:
Objection to form.
A I -- I wasn't asked to -- to make those calculations. And I would defer to other expert reports that I had an opportunity to review recently that did perform those calculations.
MS. BROWN:
Q Your opinions in this case are not dependent on whether or not --
A I think that was --
Q -- there are fragrances in Johnson & Johnson's baby powder; correct?
MS. O'DELL:
Objection.
A Sorry. Let me read that.
Sorry. Could you rephrase your question? The question that appears on the monitor is that there are fragrances in

Page 135

Johnson & Johnson baby powder, question mark.
MS. BROWN:
Q That's why it's tricky when you read the realtime. Just listen to my question. It'll be more helpful.
Your opinion in this case is not dependent on whether or not there are fragrances in Johnson & Johnson baby powder. Correct?
MS. O'DELL:
Excuse me. Objection to form.
You may refer to realtime any time you want to, Doctor.
But I object to the form of the question.
A So my -- my -- I was -- what was requested of me, again, stating for clarity, was to describe a biologically plausible mechanism for talc and all of its constituent components having a role in inflammation and progression to ovarian cancer based on -- on the information at hand.
Certainly the fact, as we've been provided later, the ex- -- the recent review of some other expert reports regarding the

Page 136

fragrances as well as asbestos, I would say my opinion now is that that information continues to support the biologically plausible mechanism presented in my report.
MS. BROWN:
Q Your opinion that chronic inflammation is a biologically plausible mechanism by which talcum powder could cause ovarian cancer is not dependent on heavy metals being present in talcum powder; correct?
MS. O'DELL:
Object to the form. Asked and answered.
A My -- my opinions are not based on -- on any singular component or constituent because the -- the available information did not scientifically test any singular components or -- or allow --
I'm not aware of any studies that examine the inflammatory or other effects of talcum powder that contained heavy metals versus did not.
MS. BROWN:
Q So, for purposes of your opinions in

Page 137

this case, for your piece of the puzzle, so to speak, it is not important to you whether or not there are heavy metals in baby powder; correct?
MS. O'DELL:
Objection to form. Asked and answered.
A No, that's not correct. I would say the presence of all of the constituent components is very important for -- from the -- from the perspective of that biologically plausible mechanism, and that includes the type of talc, the structure of the talc, you know, its -- any potential contaminants that are there, as well as the complete spectrum of other constituent components, fragrances, heavy metals.
And, of course, fragrances have their own milieu of constituent components that, again, I was not asked to comment on or describe in detail but certainly are part of the overall studies.
MS. BROWN:
Q You have a conclusion in your report on page 17, Doctor, conclusion number 2, that talcum powder products cause chronic inflammation.
Do you see that?

Page 138

A Yes.
And I would -- and then my conclu- --
Q Hold on. No question yet.
A Okay.
Q And what I want to know, Doctor, is how do you define the talcum powder products that you've listed here on page 17 of your report?
A Primarily the products that are -- when I consider the totality of everything that I've been examining, the talcum powder products, including Johnson & Johnson and Shower to Shower as, you know, I refer to those consumer products under the term "talcum powder."
Q What about other consumer talcum powder products? Are they included in your conclusions here on page 17?
MS. O'DELL:
Object to the form.
A So my -- my conclusions are based on the -- on the literature review. And, similar to our discussions regarding contaminants and the ability to quantitate those, many of the studies did not specifically delineate which product or the timing of that product.

Page 139

In contrast, some of the more recent information available specific to the constituents did meet that definition, so I would say these conclusions apply to both the specific products that I mentioned, Johnson & Johnson and Shower to Shower, as well as potentially other products. But quant- -- quantifying which study, I would have to go through study by study to answer any questions about which specific may be included.
MS. BROWN:
Q Do you include talc-containing deodorizing sprays in your definition of a talcum powder product?
A None of the literature that -- that I reviewed or can recall was limited to those deodorant sprays in terms of a -- as a study variable that I can -- that I can think of.
Q I'm not sure what you mean by that.
A So the -- the basis of this report was on the talcum powder products, and I don't recall any of the studies that delineated talcum powder as a powder versus a talc-containing deodorant spray as a -- as a variable in the study. So I

Page 140

don't know if any of the studies used -- used that. I'd have to, again, would have to review some of that information to determine if there was a -- if that was a variable in any of the given studies that are the basis of the report.
Q What methodology did you employ here in coming to your conclusion that chronic inflammation is caused by talcum powder products?
MS. O'DELL:
Objection. Asked and answered.
A Yeah. Again, to restate, similar to the earlier questions, the -- my methodology was based on standard methodology for establishing biological plausibility, which is a, in a summary, a review of the totality of the evidence and then a summary of that to establish if, based on established or -- or known or factual principles, is there a -- can -- can a mechanism described go from cause to effect in a -- again, in an evidence-supported biologically plausible manner.
There's a few references I can provide you that describe that method in a published manner, if that's helpful.

Page 141

MS. BROWN:
Q That would be helpful.
A They are -- these are our --
MS. O'DELL:
These are mine.
THE WITNESS:
Yeah.
There's a -- I can get them --
MS. BROWN:
Q Are the published methods referenced in your report, Doctor?
A No, actually, those are not.
Q Okay. How would you go about finding the published methods that contain a description of the methodology you employed in this case?
A No. It's that I was just saying that there's a published -- peer-reviewed published article that is the same as the method I used, if you -- if you wanted to review that. I didn't reference this specific paper in the report.
Q Okay. And you have a -- do you have a copy of that in front of you right now, Doctor?
A I do.
Q Okay. So let's mark that as Exhibit

Page 142

14.
(DEPOSITION EXHIBIT NUMBER 14 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q The title of the document is "Evaluating Biological Plausibility in Supporting Evidence For Action Through Systematic Reviews in Public Health."
When is the first time you reviewed this document, Doctor?
A In the last -- the last day or so.
Q Was the document provided to you by the lawyers for plaintiffs?
A Yes.
Q The document is not referenced in your report. True?
A It is not referenced. That's correct.
Q You did not review the document prior to writing your report; correct?
A That's right.
Q The document was something the lawyers for plaintiffs gave you after you had already written and authored your report; correct?
A That's correct. I provided that as an

Page 143

example of the -- of a published example of the methodology that I employed.
Q You didn't endeavor to research the scientific literature to find a published -- published example of your methodology, did you?
MS. O'DELL:
Objection to form.
A I -- it wasn't -- that wasn't what I was -- I wasn't asked to reference the methodology in my report. I was, again, asked to provide an opinion on a biologically plausible mechanism and then, since our discussion has transferred to methodology, to be complete, I wanted to provide an example of a published version of the methodology that -- that is similar to or at least describes in a summary or really in that particular paper an exemplary fashion of the criteria for biological plausibility and the methods used therein.
MS. BROWN:
Q Exhibit 14 is the product of research the lawyers for plaintiffs conducted on a published article regarding your methodology. True?

Page 144

MS. O'DELL:
Object to the form.
A No, that's not true.
MS. BROWN:
Q The lawyers for plaintiffs found Exhibit 14 in the scientific literature; correct?
A That's correct.
Q In reviewing the scientific literature, did you pay attention to the articles that classify different types of talcum powder products?
MS. O'DELL:
Object to the form.
A Could you give a specific example, and then I --
I wouldn't be able to answer without knowing.
MS. O'DELL:
Q Sure.
Do you understand that some of the talc epidemiology separates use by type of talcum powder product?
MS. O'DELL:
Objection to form.

Page 145

A Again, do you have a specific example of one of the studies so I could -- so I'd be able to accurately answer your question?
MS. BROWN:
Q Here's what I want to know. Did you look at the studies that separated deodorizing sprays from powder products from cornstarch, for example?
A Certainly in my review I made as comprehensive a review of available literature as -- as possible. And, again, if you can name a specific study or one of the references, I can confirm if that was -- if that was part of the -- my review of the epidemiology.
Q Do you hold the opinion that talcum powder-containing deodorant sprays causes inflammation?
MS. O'DELL:
Objection to form. Vague.
A So if the --
Again, I was asked to provide an opinion on the biologically plausible mechanism regarding talc and talcum powder. So, presumably, any product that contains talcum

Page 146

powder could possibly follow that same biologically plausible mechanism.
MS. BROWN:
Q Is there a certain amount of talcum powder that a product must contain to cause inflammation?
MS. O'DELL:
Objection to form.
A That wasn't something I was asked to -- to quantify, similar to the discussions we had about metals, fragrances, and asbestos.
MS. BROWN:
Q In forming your opinion that talcum powder products cause inflammation, you have not attempted to quantify how much talcum powder is in those products; is that right?
MS. O'DELL:
Objection to form. Asked and answered.
A So my -- my review included a number of studies that looked at exposure rates, and my review also included the review of some studies that did not include use frequency as well as use duration. And, so, both of those considerations in terms of my review of the epidemiology were

Page 147

undertaken, but I did not attempt to quantify those relationships specifically.
MS. BROWN:
Q Okay. So there's two different issues there that I want to ask you about. One, I want to talk to you about whether the talcum powder products you've described on page 17 of your report have a specific composition, in your mind. Okay?
Two, I want to talk to you about what you were just answering, which is is there a specific amount of the product that you believe causes inflammation.
Do you understand the difference?
A I do.
MS. O'DELL:
Objection to form.
MS. BROWN:
Q Okay. So let's start, one, with the product. In forming the opinion that talcum powder products cause inflammation, is there a particular chemical composition that you are relying on?
MS. O'DELL:

Page 148

Objection to form. Vague.
A My -- my opinions are based on the available scientific literature regarding the testing performed on talcum powder and talcum powder products.
I -- in my review of those results, I did not see a specific enumeration of any one particular chemical composition that was -- had a greater or lesser cause or effect relationship.
MS. BROWN:
Q Do you know how much talcum powder is in the Shower to Shower product?
A No. I wasn't -- I wasn't asked to quantify that, and I would defer to some of the other expert reports regarding the composition of those products.
Q Do you include cornstarch as a talcum powder product?
MS. O'DELL:
Object to the form.
A Cornstarch was included in some of the epidemiology studies, as you -- as you mentioned a moment ago.
MS. BROWN:

Page 149

Q Do you consider cornstarch to be a talcum powder product that also causes inflammation?
MS. O'DELL:
Object to the form.
A My -- my review of the literature doesn't -- I'm thinking through the available studies, and I don't recall which studies that may -- may have been a dependent variable in terms of the determination. So I -- I can't answer that. I -- I don't have the information to answer that accurately.
MS. BROWN:
Q So, sitting here today, you're not sure if cornstarch would be a talcum powder product that causes inflammation as you described on page 17?
MS. O'DELL:
Objection.
A No. So --
MS. O'DELL:
Misstates the testimony.
But you may answer if you understand the question.

Page 150

A So corn -- cornstarch and -- and talcum powder are -- are -- when I'm referring to talcum powder and talcum powder products, cornstarch, as a singular component -- or singular product, is not included in that definition.

Now, whether products that contain talc also contain cornstarch, I -- I'm not able to say.

MS. BROWN:

Q Right. And so that's my question. What about a product like Shower to Shower that contains talc and cornstarch? How have you -- what methodology have you employed to arrive at the conclusion that the Shower to Shower product causes inflammation?

MS. O'DELL:

Object to the form.

A So my -- what I was requested was to write an opinion as to the, again, the biologically plausible mechanism that exposure to talc and its constituents can lead to inflammation.

I wasn't asked to provide as to what the minimum or maximum thresholds are of any

Page 151

product or of any component of that product or constituent.

The information I was provided was the analysis of products like Shower to Shower and Johnson & Johnson's product, to evaluate the spectrum of talc and asbestos contamination in some of the constituent components, and then -- and, therefore, develop an opinion as to the -- whether or not that those products are supported by the same mechanism that I developed the opinion on, meaning they have the constituent components to cause inflammation.

MS. BROWN:

Q You have not made a determination of a particular amount of talcum powder that is required to be in a product for it to cause chronic inflammation; correct?

MS. O'DELL:

Object to the form.

A I wasn't asked to provide such an opinion.

MS. BROWN:

Q Your opinion that talcum powder products cause chronic inflammation is not based

Page 152

on knowledge of how much talcum powder is actually in the product; correct?

MS. O'DELL:

Objection. Misstates his testimony.

A Again, not a -- it wasn't part of -- it wasn't an opinion I was asked to provide.

The -- the only -- or, I should say, a -- a study that looked at the -- summarizing the epidemiology literature that I reviewed, some of those studies had a duration and component as far as general talcum powder and talcum powder product use.

MS. BROWN:

Q And I want to --

A I don't --

MS. O'DELL:

Excuse me. Let him finish.

A I was -- I was going to say I don't recall those quantitating the percentage of talcum powder in a -- in a given product in the study.

MS. BROWN:

Q Right. And, so, you're getting a little into the second question, which I do want

Page 153

to talk about, which is how much people are exposed to.

But sticking with just what's in the product, have you made a determination that there is a threshold amount of talcum powder that is required to be in a product before you can conclude that that product will cause chronic inflammation?

MS. O'DELL:

Objection to form. Asked and answered.

A I -- again, I wasn't asked to provide that -- that threshold opinion.

MS. BROWN:

Q And understanding whether or not there is a threshold of how much talcum powder has to be in a product to cause inflammation is not necessary for you to opine that talcum powder products cause chronic inflammation?

MS. O'DELL:

Objection. Misstates his testimony.

A So my -- my use of the terminology "talcum powder products" includes the product and all of its constituent components, which would be, as we earlier discussed, talcum powder,

Page 154

fragrances, and any contaminating substances, such as asbestos or -- or heavy metals.

And, so, therefore, to -- to more -- to answer -- to be able to answer your question accurately, we would -- I think we would have to have some discussions as to the type of talcum powder and the level of exposure to be able to answer that regarding my opinion in terms of level.

You know, the -- to clarify, the -- during this research and the -- and having the opportunity to review much of the literature in talcum powder, it's a -- it's a fascinating field because it is similar to asbestos. It appears that the diversity of products and the diversity of talc sources are like having a thorn bush with different size thorns, and, depending on the constituent components, you know, those thorns are bigger or smaller or otherwise. And -- but my opinion is based on the fact that the presence of any of those thorns is sufficient to cause some inflammatory response.

MS. BROWN:

Q Does a talcum powder product with 10

Page 155

percent talc cause chronic inflammation, in your view?

MS. O'DELL:

Object to the form. Incomplete hypothetical.

A I -- I don't have the information to answer that.

MS. BROWN:

Q Does a talcum powder product with 50 percent talc cause chronic inflammation, in your view?

A Again, I don't have the information to answer that.

MS. O'DELL:

Object to the form.

MS. BROWN:

Q Is it necessary for you to determine the level of talc in a product before determining that it can cause chronic inflammation?

MS. O'DELL:

Objection. Asked and answered.

A No. My -- my -- so my opinion was asked to answer the question of can -- is there a biologically plausible mechanism from talc

Page 156

exposure to inflammation to the initiation of core progression of cancer. And that's -- that's been the focus of my opinion.

MS. BROWN:

Q Have you attempted to quantify talc exposure as it relates to individuals?

A No, I have not.

Again, my -- my opinions are primarily limited to the -- to the biological mechanism.

Q Well, isn't that dependent, though, on how much talc a person is exposed to?

MS. O'DELL:

Objection.

A No. Again, separating the -- so the question of the mechanism is --

Can an exposure result in a mechanism is separate from how much of an exposure is required to cause that mechanism.

MS. BROWN:

Q So you've identified two questions for us. One, can exposure result in a mechanism. Correct?

A (Nods affirmatively.)

Q And, two, how much of an exposure do

Page 157

you need to produce a mechanism. Correct?

MS. O'DELL:

Objection to form.

A Correct.

MS. BROWN:

Q And, in this case, you have answered question number one, can exposure to talc cause chronic inflammation. Correct?

A So my -- yeah. My -- my report details the -- that opinion regarding a biologically plausible mechanism.

Q You have not, in this case, answered question number two, which is how much exposure to talc is needed to cause chronic inflammation. Is that right?

MS. O'DELL:

Objection to form.

A I wasn't asked to provide such a mechanism or such a -- such an opinion.

Part of my review included some of the epidemiology studies that examine that question, but I certainly would defer to the -- the number of -- of epidemiologists who are -- who are providing testimony in this case, rather than try

Page 158

and paraphrase or opine on their work.
MS. BROWN:
Q Do you believe --
MS. O'DELL:
Excuse me. We've been going about an hour and 15 minutes. I'd love to take a break in the next two or three minutes and --
MS. BROWN:
It will probably take me a little longer than that, but I'm mindful of the time, and I'll just finish this subject and take a break --
MS. O'DELL:
Well, Dr. Levy, would you like a break now?
THE WITNESS:
I think we can finish this subject.
MS. BROWN:
Thank you.
THE WITNESS:
I -- I'd rather conclude it than break it up.
MS. BROWN:
Q So, Doctor, as it relates to how much

Page 159

talc is needed to cause inflammation that can cause cancer, that wasn't what you were asked to figure out in this case. Is that right?
MS. O'DELL:
Objection to form.
A No. Well, I -- I was -- I was asked to provide a review of the literature in terms of talc exposure and inflammation and, in that review, identified a number of studies that examined some relationships to dose.
But I -- as you -- as you see in my conclusions, none of them speak to dose or duration in terms of that -- of that mechanism.
MS. BROWN:
Q You are not offering an opinion in this case, Doctor, that perineal use of talcum powder exposes an individual to enough talc to cause chronic inflammation than can cause cancer; correct?
MS. O'DELL:
Objection to form.
A My review of studies that attempted to answer that specific question found a -- or a number of studies, both -- or a number of

Page 160

epidemiology studies found that conclusion and, as -- as reviewed in the report, you know, found an increased risk with increasing -- increasing exposure appears, with the current knowledge in the literature, to increase risk. But my opinion was not to further quantify or further describe that.
MS. BROWN:
Q Many of the studies you looked at did not show a dose response; correct?
MS. O'DELL:
Objection to form.
A The limitation of several of the studies I reviewed was that they did not examine a dose response, so that, therefore, the study was unable -- unable to make that conclusion because they didn't look.
MS. BROWN:
Q And some of the studies that did attempt to look at duration and/or frequency did not show a linear dose response. Correct?
A I would have to look at the specific studies. But in -- in summary, studies that did look at dose response, particularly more recent

Page 161

studies with larger numbers of participants, the meta-analysis studies, found a significant relationship between duration of use as well as frequency of use in terms of their -- their risk ratios.
Q And you are not going to offer the opinion in this case that a woman using Johnson's Baby Powder products perineally is exposed to enough talcum powder to cause chronic inflammation that can cause cancer. True?
MS. O'DELL:
Object to the form.
A I -- I wasn't asked to -- to provide that opinion.
MS. BROWN:
Q And so, as such, you haven't attempted to quantify how much talcum powder, as used perineally, might get to the ovary. Is that fair?
A Again, wasn't -- wasn't asked. I was able to review some of the literature that is -- appears to be long -- longstanding, well established over the last greater than 40 years that show a clear -- and I believe the FDA

Page 162

statement is -- is describing it as inarguable -- that talc can migrate either from perineal exposure or even from inhalation exposure and be found in the ovary.

A quantitation of how much exposure is required for that migration to occur and -- or how many times of exposure that migration needs to occur, I think it's been a fairly wide diversity of -- of studies on that subject.

And, so, based on that, I'm not able to offer an opinion as to a minimal or maximum dose required to get there, other than -- but, instead, state that there is enough evidence to say factually that migration through the -- or through at least two mechanisms of exposure, talc can be found in the ovary. And I would suggest that -- or I'm not aware of any study that quantitates that further.

Q Is it essential to your opinion that talc causes chronic inflammation that can lead to ovarian cancer that some amount of talc be present in the actual ovary?

MS. O'DELL:

Object to the form.

Page 163

A So my -- my -- my opinion regarding the biologically plausible mechanism, again, does not rely on duration of exposure or amount of exposure.

So, therefore, I would -- I would answer your question directly that it would be no, it does not -- it would not necessarily require talc to be present at the ovary at any given time point for there to be the potential that she had some inflammatory injury due to talc exposure at a previous time.

That would, of course, be two different questions, one being effect of exposure and second question being is there clearance of that exposure over time if use is discontinued.

So that's, again, two different -- two very different scientific studies would be -- would be necessary.

MS. BROWN:

Q And you have not undertaken either of those studies. Is that fair?

A That's fair.

Q And -- but essential to your theory, though, Doctor, at some point, some amount of talc has to reach the ovary for the chronic inflammation to occur. Is that right?

MS. O'DELL:

Objection.

A Not -- specific to your question, chronic inflammation, no, not necessarily.

MS. BROWN:

Q Is it your opinion in this case, Doctor, that a woman can develop ovarian cancer from chronic inflammation from talc without any particle of talc ever reaching the ovary?

MS. O'DELL:

Objection to form.

A No, I didn't -- I -- I certainly did not make that statement. And the --

Again, restating the -- this summary of my -- my opinion, that the biologically plausible mechanism for talc exposure to inflammation to cellular damage and then potentially creating the correct environment is based on evidence showing talc exposure in the ovary.

MS. BROWN:

Q Okay. So critical to your opinion, then, some talc has to get to the ovary at some time; right?

A Well, the -- again, the -- my opinion is not based on how talc migrates or -- or when it can migrate. It's simply based on the, again, that biological premise, that exposure to talc.

So I wasn't asked to opine whether or not talc exposure in a neighboring tissue could cause enough of an inflammatory response to affect the ovary.

So there is the, certainly, the uninvestigated secondary effects that perhaps talc did not -- is not necessary or -- and required to get to the ovary to cause that effect. I'm -- I'm just not aware of any studies that have made that delineation of talc exposure to neighboring or surrounding organs.

There is limited or some suggestion regarding the inflammatory response related to talc exposure in the lung that suggests that any talc exposure causes an inflammatory response. Again, but I can't point you to evidence that would take that inflammatory response and tie it specifically to ovarian cancer.

So, again, my answer is there is not

Page 166

enough evidence to -- to support nor refute that any talc exposure can lead to an increased risk of ovarian cancer. What I do know from my review of the literature is the studies that looked at that specific exposure --

And, to be clear, none of the epidemiology studies in humans quantitated the amount of talc reaching the ovary. It was simply the exposure and the -- and the perineal use of talc. So I think any discussion about how much did it reach the ovary and how long was it in the ovary is all hypothetical.

Q Why don't we go off the record and take a break.

Thank you, Doctor.

VIDEOGRAPHER:

Going off the record. The time is 11:51 a m.

(LUNCH RECESS.)

VIDEOGRAPHER:

We're back on the record. The time is 12:52 p m.

MS. BROWN:

Q Welcome back, Doctor.

Page 167

You were asked in this case to assess whether perineal use of talcum powder products induces a biologically plausible mechanism or mechanisms that result in ovarian cancer. Correct?

A Correct.

Q And define for us, if you will, "biologically plausible mechanism" as you used it in that sentence.

A Excuse me. A mechanism that is biologically plausible, I mean that it is supported by either well-established biological facts or supported by at least a single line of evidence in published literature -- you know, generally speaking, peer-reviewed literature but certainly not limited to that -- where when you take -- when you consider the totality of the mechanism, that, essentially, each of the steps makes sense and is -- is supported by -- through either direct or indirect observations.

Q Okay. And, in this case, as it relates to talcum powder, do you believe that the biologically plausible mechanism of chronic inflammation causing ovarian cancer is supported

Page 168

by well-established biological facts?

A I would say the -- that chronic inflammation as a component of causing ovarian cancer is well established by biologically plausible facts.

Q And what are those facts?

A I think a number of studies that include the, first, the -- that talc or talcum powder causes inflammation. These exist in a number of forms, including very recent -- recent research by Dr. Saed, as we were -- touched on a little bit earlier in the -- in his paper, as well as classical studies with talc pleurodesis where there's -- you know, the fundamentals of that treatment is the inflammatory response caused by talc.

Q Uh-huh.

A And, so, that would be the -- some of the -- two examples of where factual information or at least observations that are supportive of -- of that information, you know, being considered as a bio- -- part of a biologically plausible mechanism.

Q You would agree, Doctor, that not all

Page 169

inflammation causes cancer; correct?

A I would say inflammation is not singularly responsible for cancer. However, I would clarify that the progression from cellular transformation to malignant cancer, at least with our current understanding of cancer biology, appears to have an inflammatory requirement, meaning that all cases of chronic inflammation don't necessarily cause cancer. However, our understanding of malignant transformation appears to have, universally, an inflammatory component.

Q Okay. You would agree, though, that not all types of inflammation that the body experiences is inflammation that will lead to cancer. Correct?

MS. O'DELL:

Object to the form.

A So I would -- taking a step back and -- and -- or to orient us to some of the basis of my opinions and some statements on general cancer biology --

MS. BROWN:

Q Well, let's start with just the question, though, Doctor.

Page 170

A Okay.
Q Okay. Let's just keep it to an answer to the question. And then if you need an opportunity to make another statement on the record, that's fine.
MS. O'DELL:
Excuse me. Just object to the direction of the witness.
Dr. Levy, you can answer a question however you'd like.
MS. BROWN:
Q And, just to orient you, Doctor, what I'm after, the question was: Not all inflammation that takes place in the body is inflammation that leads to cancer; correct?
MS. O'DELL:
Object to the form.
A So that, yeah, it's really too general a question. So you're -- you're -- what you're asking is does all inflammation have the potential to have -- have a relationship to cancer, and the answer to that is -- is yes, it does.
Now, does every inflammatory response

Page 171

directly cause cancer? And that's a question that I would say would be reasonable to -- in layperson's terms, in terms of general inflammation, is unlikely.
But there -- their distinction between -- is -- you know, stated simply, is inflammation is a -- by our current knowledge of cancer, is a necessary component of cancer progression. That does not equate to all inflammation causing cancer.
MS. BROWN:
Q Does acute inflammation cause cancer, in your mind, Doctor?
A It is a component of the cancer progression process. And, so, in my -- to provide a simplistic distinction between them is a --
Acute inflammation which results in either an inflammatory response or direct cellular insult or injury can be viewed as having a -- causing cellular damage that results in -- in cellular transformation.
Now, that is not sufficient for that -- for those transformed cells to then go on to

Page 172

cause cancer. The -- you need a contribution of other factors. And what those factors are is -- some are understood. Some are areas of active research.
In the -- in the specific case of ovarian cancer, it does appear, given the late- -- given the observations about latency period, that some level of chronic inflammation appears to be critical, but there is no definition of it being required to then having acute inflammation, again, in summary, causing cellular damage and then chronic inflammation providing a -- a supportive environment for that transformation.
And, again, I'm -- I'm generalizing, which, as we discussed earlier in the day, cancer is very complex, and so we have to be cautious with generalizations.
Q Talc pleurodesis is a medical procedure by which talc is injected into the pleura; correct?
A Correct.
Q And it is done that purposefully to elicit an inflammatory response. Correct?

Page 173

A That's correct.
Q And have you looked in consid- -- forming your opinions in this case at the body of epidemiology that has followed folks who received talc pleurodesis to see if they developed cancer?
MS. O'DELL:
Object.
A Somewhat, yes.
MS. BROWN:
Q And are you familiar with the findings of those studies that talc, when injected directly into the pleura for the purpose of causing inflammation, had not caused cancer?
MS. O'DELL:
Object to the form.
A I would disagree with your conclusions. And, in fact, the literature I reviewed has, I think, two fundamental concerns. One is the time period that these patients were followed post pleurodesis, and the other that there -- there have been at least one report, perhaps two -- I would have to review to make sure I'm speaking accurately -- where there was indeed a asbestos-like response in the formation of a

Page 174

mesothelioma-like event in the -- in the -- in the pleural space following talc pleurodesis.

However, you know, taking a step back, given the relative rarity of that as a procedure, particularly today, I think drawing conclusions from that as its -- as its relationship to cancer would be difficult, but I -- I do think fundamentally the -- my use of that as an example was not necessarily to tie talc specifically to cancer. It was more to state that it's well established that platy talc individually as it -- used in those procedures causes an inflammatory response. And so, you know -- and that is the primary reason I used or reviewed that literature for that purpose.

MS. BROWN:

Q Is it your opinion, Doctor, that talc pleurodesis leads to cancer?

MS. O'DELL:

Object to the form.

A It is my opinion that talc pleurodesis creates an environment supportive of cancer. And whether or not some number of individuals may progress, could progress or have progressed to

Page 175

cancer is -- you know, is -- is of limited knowledge right now.

MS. BROWN:

Q What scientific support do you have for your opinion that talc pleurodesis creates an environment supportive of cancer?

A Oh, just that it causes an inflammatory response. And, as we've been discussing, there is ample evidence surrounding the role of inflammation in cancer. There's a -- you know, in a number of both reference studies and I think generally, I would -- I would state that it's a generally accepted fact in cancer biology.

Q What scientific support do you have for your opinion that talc pleurodesis patients later can and do develop cancer?

MS. O'DELL:

Object to the form. Misstate his testimony.

A I'd have to review my -- review some of the literature. And I can take a look if we want to pause for a moment.

But there was -- I recall one study involving talc pleurodesis that was maybe

Page 176

mid-'80s to early '90s. I'd have to, again, have to review that --

I gave that specific example of a patient or cohort of patients that were found to have, again, asbestos-like effects in the lung leading to, at least in a case or more than perhaps more than one case, a mesothelioma-like effect like we -- like I just mentioned.

But, again, to point you to the exact reference, I'd have to review.

MS. BROWN:

Q Are you relying on that reference in forming your opinions in this case?

A No. Specifically -- again, to restate the -- my description of the pleurodesis process was to support the early part of the biological mechanism that talc causes inflammation. So that -- and, so, in the lung as a tissue, that progression to cancer is -- is -- I think is a -- is a -- is a supportive observation to the -- to my overall principle. But, again, it's a separate -- separate exposure type, certainly a very different dosing, potentially, and, again, a very different patient, or the patient is a very

Page 177

different individual in the sense that they obviously have reasons for going through the talc pleurodesis which are -- which are -- which are potentially compounding to the overall phenotype.

Q Have you endeavored to quantify the difference between exposure to talc from pleurodesis versus perineal use of cosmetic talcum powder products?

MS. O'DELL:

Object to the form.

A I have -- I have not attempted to delineate those two simply from the perspective that, again, to the biological mechanism, the initial premise is talc causes inflammation. And when I examined literature to look for evidence of that historically, talc pleurodesis is one example of inflammation. There's now others, and there's, subsequent to that, there's been a -- now a number of -- or, you know, probably a --

Dr. Saed is one example of a reasonably comprehensive molecular study examining specific inflammatory markers tied specifically to cellular exposure to, in the case of that paper,

Page 178

specific products, you know, such as the Shower to Shower and the -- and baby powder.
MS. BROWN:
Q Do you believe the inflammation caused by talc pleurodesis is chronic inflammation that leads to cancer?
MS. O'DELL:
Objection to form. Asked and answered.
A Again, I believe the inflammatory response to talc exposure, which would include talc pleurodesis, induces an inflammatory response that would be supportive of cancer development and/or progression.
MS. BROWN:
Q And what scientific literature other than the one study you just referenced for us do you rely on for your opinion that talc pleurodesis induces an inflammatory response that would be supportive of cancer development and/or progression?
MS. O'DELL:
Object to the form.
A All my -- my opinion is based on connecting two basic concepts. Talc exposure

Page 179

causes inflammation. Inflammation has a significant role in cancer development.
And, so, as far as -- each of those is supported by individual -- individual studies, and -- and now -- as I mentioned, there are now studies that directly tie those together in observation.
MS. BROWN:
Q What is the scientific basis for your support that talc exposure causes the type of inflammation that has been linked to cancer?
A The most recent is the Saed publication that we discussed and -- or at least has been mentioned. In that study, looking at -- there was a assessment and, in some cases, a quantitation of the specific molecular markers for inflammation that were induced, and many of -- some of those markers are shared with known markers for -- for cancer progression, such as CA 125, as well as others.
Q Are you referring to Saed's 2018 paper, Dr. Levy?
A Yes.
Q And you formed the opinions that talcum

Page 180

powder products cause chronic inflammation in your November 2018 report before having seen the Saed paper from 2018; correct?
MS. O'DELL:
Object -- object to the form.
Misstates his testimony.
A The -- so, as we discussed -- we discussed earlier, I had seen abstract information as well as earlier publication from Dr. Saed's group and that the current 2018 paper, while not necessary for the opinions described in the report, certainly support those opinions, given that it was a direct assessment of specific products, specific -- in specific doses applied to cellular material and then measurements for inflammation made directly on that material.
So while that particular study was not --
And, again, the -- the earlier studies that were used to inform the 2018 paper were certainly used in this report and referenced the --
And I'm just recalling when. Or if we've refer- -- had the opportunity to reference

Page 181

the --
Yeah. So we reference primarily the abstracts and then, again, as well as some of the other Saed work, which is the foundation of the directed studies that are described in the Reproductive Sciences paper that is Exhibit 12.
MS. BROWN:
Q Do you know that Dr. Saed is a paid expert for the plaintiffs' lawyers in this litigation?
A I am aware. Yes.
Q Have you considered that fact in evaluating Dr. Saed's work?
A I did.
Q Other than Dr. Saed's work from 2017 and 2018, what evidence are you relying on to support your opinion that talcum powder produces the type of inflammation that can lead to cancer?
A There has been -- looking through the -- there's the Buz'Zard and Lau, 2007. We were discussing the Hamilton -- Hamilton paper in terms of immune response but then, more specifically, the NTP reference in 1993. And in those cases, that was either looking at increases

Page 182

in reactive oxygen species generation --
THE COURT REPORTER:
Wait a minute. You have to slow down when you read, please.
MS. O'DELL:
You may continue.
A Just to -- before I left off, I think, in those mentioned references, the reactive oxygen species generation, increased cell proliferation, and the use of -- in the specific case of Buz'Zard and Lau, was looking at the transformation in human ovarian cancer cells that were treated with talcum powder -- sorry -- human ovarian cells treated with talcum powder.
MS. BROWN:
Q Other than Buz'Zard, Hamilton, and NTP, is there anything else that you are relying on to support your opinion that the inflammation caused by talcum powder is the type of inflammation that causes cancer?
A So there's additional references mentioned in the report; Gates, Belot, Harper and Saed. And then, in addition to that, there was a --

Page 183

Make sure I'm referring to the right one.
So those were the -- those were the primary references. And then, of course, there were supporting materials and other earlier-cited work.
But for the opinion regarding the type of inflammation that is caused by exposure to talc and as far as its specific relationship to cancer, there's -- there's -- I would point to the, at least in the Saed work, the specific quantitation of a very well-known tumor marker, CA 125, also known as mucin-16 elevation in that work, and then, in the case of Gates, some of the fundamental glutathione S-transferase has been associated or has been observed as a higher risk.
And, so, that would -- those would be some examples.
Q Are you aware of any animal study, Dr. Levy, that shows the inflammation caused by talcum powder causing precancerous changes?
MS. O'DELL:
Object to the form.
A I would have to review the -- a few of

Page 184

the details, and I -- there -- I am aware of -- mentioned earlier the Woodruff or Woodford, the earlier 1971 paper where I couldn't remember the author, is one of the earliest studies that I came across that had -- it has an animal model study.
MS. BROWN:
Q Doctor, is it your testimony that --
First of all, do you think it's -- that in opining that there is a biologically plausible mechanism by which talcum powder causes chronic inflammation that can cause ovarian cancer, is it necessary, in your mind, to be able to show in animals that talcum powder does just that?
A That talcum powder causes inflammation?
Q That causes ovarian cancer.
A No, I don't -- I don't think that that's -- that's certainly not a requirement. And the reason I -- the reason I give that answer is -- is quite simple; that there is a wide diversity of animal model studies that have not been able to mimic specifically or correctly human cancer for both -- both from a detection and most often from a treatment perspective,

Page 185

meaning that, fundamentally, humans and most -- or at least the animal systems used as -- in scientific modeling are different. Some of their differences are due to different pathways, and others of the differences are due to actually, you know, fundamental immune system differences.
Q The Hamilton article that you identified for me, we marked earlier in the deposition as Exhibit 7. Do you recall that?
MS. O'DELL:
Counsel, would you mind just placing the exhibits by the witness so he can refer to them as he'd like, please.
A Yes, I recall this.
MS. BROWN:
Q And you would agree with me, Doctor, that the Hamilton study that we discussed this morning concluded that there were no neoplastic changes in the animals that were injected with talcum powder; correct?
MS. O'DELL:
Object to the form. Asked and answered.
A No. No, I -- I wouldn't agree.

Page 186

MS. BROWN:
Q What evidence in Hamilton, Doctor, are you relying on to support your position that Hamilton showed neoplastic changes in animals injected with talc?
A Well, I'm not -- I'm not stating that Hamilton specifically showed that.
What I'm stating is that -- that there is a Hamilton study as an animal model system to make the conclusion that, in this animal model system, that talc or talcum powder does not -- or that causes or does not cause ovarian cancer is not -- it's -- it is -- it has limitations.
And, as we discussed a bit earlier, the two limitations are the very limited time points of the animals. And if we look at the relative and observed time points that we know now, as far as latency period, these are well short of those -- of those periods, even by rat standards, and then the number of treated animals is relatively small at ten. So the...
Q Doctor, do you rely on the Hamilton article to support your opinion that talcum powder produces chronic inflammation that causes

Page 187

ovarian cancer?
A No, I don't rely -- again, I don't rely on any -- there's not a reliance on any singular article.
Q Did not mean to suggest that, Doctor.
I asked you for the scientific support that you have for the opinions you're giving in this litigation, and one of the articles you identified was the Hamilton article. Correct?
A Uh-huh. Yes.
Q And I -- and this Hamilton article, as we discussed, at page 103, found no evidence of neoplasm in the rats injected with talc. Right?
A They -- I -- I don't -- they did not -- I don't recall seeing a description of neoplasm in the Hamilton article.
Q Page 103, second column, begins with "No evidence."
A "No evidence of cellular atypia."
Q Uh-huh. "And concludes that in no ovary was there any evidence of frank neoplasia"; right?
A Yes. That's what's written in the paper.

Page 188

Q So this article looked at talc that was injected into animals and found no evidence of changes that lead to cancer. Correct?
MS. O'DELL:
Objection to form.
A Over the time period that they -- that the study was performed, they did -- they did not -- they did not report, and, in fact, as you said, their statements are "no evidence of cellular atypia or mitotic activity."
MS. BROWN:
Q So in opining, as you do in this case, that talcum powder can biologically induce chronic inflammation that causes ovarian cancer, what methodology did you employ to consider the findings of the Hamilton article?
A Well, I considered the findings of the Hamilton article, as -- as referenced in the report, primarily showing that talc has an inflammatory or an immune response. And that was the primary inclusion of the -- of the Hamilton paper.
Q Not all inflammatory or immune responses lead to cancer; right?

Page 189

MS. O'DELL:
Objection. Asked and answered.
A As -- as we discussed, not -- not all inflammatory responses have been shown to conclusively lead to cancer. And, so...
MS. BROWN:
Q And Hamilton does not support the opinion that the type of inflammatory response that talc causes is the type that causes cancer. Fair enough?
MS. O'DELL:
Object to the form.
A No. I would say that's unfair. Because, again, the limitation of the Hamilton study at the time it was performed was -- is a very short timeline. So there is -- it is an incomplete study in the sense that there is certainly the possibility that the first aspect or the first event that we're -- that we've been discussing in cancer biology, the cellular damage to lead to transformation, could have occurred in some of the rat tissues but had not progressed enough or had -- or had taken hold enough to cause or to have that be detected in this

Page 190

particular study performed in the early '80s.
And, furthermore, rat -- the rat model for human cancer, since this study has been in other cases, has some limitations as it relates to how applicable it is to the human condition.
MS. BROWN:
Q The NTP study that you identified as supporting your opinion, Doctor, that also does not show evidence of neoplastic changes; is that right?
MS. O'DELL:
Object to the form.
Doctor, please feel free to refer to the study if you need to.
A Yeah. I'll do that now.
(DEPOSITION EXHIBIT NUMBER 15 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q Doctor, we'll mark as Exhibit 15 to your deposition the NTP study to which you were referring.
A Uh-huh.
Q And this study, as well, does not show evidence of neoplastic changes.

Page 191

MS. O'DELL:
Object to the form.
Do you have a copy for me?
It's what number?
MS. BROWN:
Fifteen.
A I think the -- the important distinction in this particular study is this was an aerosol-based -- based study. It certainly was longer than the Hamilton but was -- was not a study that mimics the perineal use of talc.
MS. BROWN:
Q And, so, as it relates to your opinion in this case, Doctor, that talc induces a chronic inflammation that can lead to ovarian cancer, the NTP study does not support that, does it?
MS. O'DELL:
Object to the form.
A I would say the study does support my opinion regarding talc and its role in inflammation. And if we refer to page 6 within the first -- the first paragraph, beginning with "Accumulations of macrophages."
MS. BROWN:

Page 192

Q Did you review, Doctor, the --
And -- and what about the findings of NTP support your opinion?
A Well, first, the inflammatory response, given the evidence by the accumulation of macrophages, and then, secondly, that in the female rats, the incidences of alveolar and bronchial or adenoma, carcinoma, and adenoma in the 18-milligram-per-meter group were significantly greater than those of controls.
Q So did you consider the FDA's findings as it relates to the evaluation of the NTP study?
MS. O'DELL:
Object to the form. Vague.
A Which -- which FDA?
MS. BROWN:
Q Have you considered, in connection with this case, the FDA's response to the 2014 citizens petition?
A Yes. That's familiar. And if I recall correctly --
Or do you have -- is that handy?
Q We'll mark that as Exhibit 16, Doctor.
(DEPOSITION EXHIBIT NUMBER 16

Page 193

WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q The reason I want to talk to you about this is it contains a review of the NTP study we were just discussing.
First of all, did you consider this document in connection with your opinions in this case?
A Yes, this document's familiar.
Q Okay. And do you recall that a cancer prevention coalition wrote the FDA requesting that a warning label be placed on talcum powder products?
A Yes.
Q And do you recall, as evidenced on page 1, the FDA reviewed the data as it related to that question?
A I -- I recall that the FDA reviewed the data and determined that it was insufficient, and they did not identify any new compelling literature at the time. But this was in 2014.
Q And the NTP --
MS. O'DELL:
Excuse me, counsel.

Page 194

Were you finished? If you're finished, that's fine. I just didn't know if you completed your --
A I'm just reading. There was one other -- I recall --
MS. BROWN:
Q Doctor, the NTP study that you pointed us to was from 1993. Is that right?
A I believe that's correct.
Q All right. And one of the things that the FDA did in this letter of 2014 is reviewed that study; correct?
A Yes.
Q And I'll direct you to page 3 of 7. And what the FDA concluded was that the study lacked convincing scientific support because of serious flaws in its design and conduct.
Do you see that?
MS. O'DELL:
Where are you reading? Sorry.
MS. BROWN:
Page 3. Page 3.
MS. O'DELL:
Oh. Page 3. Sorry. I thought you

Page 195

said page 2. I'm sorry.
MS. BROWN:
Q Do you see that, Doctor?
A Starting with --
Q Bottom of page 3 --
A -- under toxicology findings?
Q So, to orient us here, Doctor, you pointed, as evidence of support of your opinions in this case, to the NTP study. Right?
A Correct.
Q And the folks who wrote to the FDA requesting a warning on talc, they, too, pointed to that study; right?
A Yes.
Q All right. And, so, the FDA reviewed that study and, in the letter denying the citizens petition, included its critique of that study; correct?
A Correct.
Q And one of the things the FDA concluded was that the study had serious flaws. True?
MS. O'DELL:
Objection to form.
A I don't -- do you -- I don't see where

Page 196

the FDA claimed serious flaws.
MS. BROWN:
Q At the bottom of page 3 --
A I see.
Q -- the sentence that begins, "However, this study lacks convincing scientific support because of serious flaws in its design and conduct -- and conduct."
Do you see that?
A I do.
Q And one of the things the FDA points to is that the investigators used micronized talc instead of consumer grade talc, resulting in the experimental protocol not being reflective of human exposure conditions in terms of particle size.
Do you see that?
A I do.
Q Have you made a determination in this case, sir, about the size of the particles in talcum powder products?
A I -- I've not made that distinction. And --
Q There's --

Page 197

A And, furthermore, I think the -- importantly, the -- the flaws that the FDA points out are, you know, not in disagreement with our -- with our discussions surrounding both the inflammatory response and then some of the results there. I don't -- I don't see as a concern --
In fact, the -- it appears the FDA does not disagree with the observation of the evidence of carcinogenic activity in the non-asbestiform talc. I think they --
I share --
Q Let's focus back on the question, Doctor.
MS. O'DELL:
Excuse me. Let him finish his answer. He's not finished.
A So, the, you know, the serious flaws were the, I think, in this case, the specific inclusion of nonasbestos talc and use of micronized talc instead of consumer grade. So I think in that -- in that sense, it's not surprising that it had a different -- perhaps a different response than may be observed with

Page 198

consumer products or talc that have -- may have contaminants, whether it be asbestos or other.
MS. BROWN:
Q Do you remember the question I asked, Doctor?
A Perhaps it would be helpful to restate.
Q I think, probably.
I asked if you had made a determination in this case about the size of the particles in talcum powder products.
A I -- so as far -- a determination, no. I would -- I would say I have had an opportunity to, you know, review or become more educated in the diversity of talc products and the interesting geographic relationship to different size particles and -- in the presence or absence of asbestiform particles in talc, which was a, you know, fascinating area to become educated in.
As far as examining that in each of the individual studies, I certainly was able to pay attention to earlier or later studies as it applied to when there was a specific description of the talc, such as in the NTP study where there -- that was one of the few that had a

Page 199

specific determination.
But I was basing my opinions on the general behavior, summarized behavior of talc based on the available evidence.
Q In forming your opinions in this case, Doctor, have you concluded that a particular route of exposure is more likely when women are using talcum powder products perineally?
MS. O'DELL:
Object to the form.
A Certainly it would seem logical that the route of talc exposure would be related to the area that the talc is used.
MS. BROWN:
Q As such, do you believe and have you assumed for purposes in your -- of your opinions in this case that talc more likely migrates from the perineum to the ovaries, as opposed to talc being inhaled and then traveling down to the ovaries?
A The evidence I've seen would suggest that that migration that you described from the perineum through the vagina into the fallopian tubes into the ovary is certainly far more likely

Page 200

when -- when used in the perineum compared to inhalation.
But I have not seen a study that tried to distinguish that in terms of having an exposed group who inhaled talc only and then looked for evidence of the presence in the ovary.
Q Back to the FDA document we were discussing, Doctor, the FDA's critique of the NTP study continues on page 4, where the FDA identifies that the investigators conceded they have problems with the aerosol generation system and that the study did not include positive and negative dust controls.
Did you consider those critiques in evaluating the NTP study in this case?
MS. O'DELL:
Object to the form.
A Well, I -- I certainly considered -- you know, considered them in -- as -- as I would consider any -- any other evidence or opinion on -- on these relevant subjects.
MS. BROWN:
Q The FDA went on to conclude, Doctor, that, in light of the shortcoming, a panel of

Page 201

experts at the 1994 ISRTP/FDA workshop declared that the 1993 NTP study has no relevance to human risk.
Do you share that conclusion?
MS. O'DELL:
Object to the form.
A I do not. And I think, importantly, you know, even there at the bottom of page 4, their point number 4 saying a cogent biological mechanism by which talc might lead to ovarian cancer is lacking.
MS. BROWN:
Q Uh-huh.
A I believe, as we're discussing today, subsequent research and subsequent studies have -- and including my report, have helped define that plausible biological mechanism which -- by which talc may lead to ovarian cancer.
Q In answering my question, Doctor, you pointed to a different portion of the same page we were discussing; correct?
A Correct.
Q And what you pointed to was the FDA's

Page 202

conclusion here in 2014 that a cogent biological mechanism by which talc might lead to ovarian cancer is lacking. Correct?
MS. O'DELL:
Object to the form.
A I -- I would disagree in the general nature of your statement and clarify it by saying the FDA found a lack of that mechanism based on the submitted literature of the citizen petition.
MS. BROWN:
Q So do you understand, Doctor, in evaluating the FDA's response, that they, in fact, did their own investigation in addition to the literature that was provided to them at the time?
MS. O'DELL:
Objection. Misstates the record.
A Well, my reading of it, it says they -- that their -- that the scientific literature considered was submitted in support of both citizen petitions. And...
MS. BROWN:
Q Are you finished, Doctor?
A Yes. I was just looking to see if

Page 203

there was a notation about further --
Q I'll direct you, Doctor, to page 4, the second full paragraph that begins "In addition, the FDA stated."
"In addition, we reviewed relevant toxicity literature (consisting of 15 articles from 1980 to 2008) not cited in your petition to determine if there was additional support at this point in time for your suggested warning label."
Do you see that?
A I do.
Q And, based on the FDA's review of all the literature that they investigated at the time, they concluded that a cogent biological mechanism by which talc might lead to ovarian cancer was lacking. Right?
MS. O'DELL:
Objection to form.
MS. BROWN:
Q That was their conclusion; correct?
A Yes, as written, that was their -- that was the FDA's conclusion.
Q And you, Dr. Levy, disagree with that conclusion; correct?

Page 204

A I -- I disagree with the -- or I -- I have found, based on a review of the literature, that there are now additional supporting studies that would -- that would refute some of these conclusions of -- by the FDA review.
Q And explain to us, then, Doctor, what methodology you employed or what research you conducted to reach a conclusion different from the FDA's conclusion in 2014.
A I think, similar to what the FDA described, my review is of the literature now, you know, through 2018, examining the available information regarding inflammatory response to talc and then talc exposure as it relates to -- to the initiation of progression of cancer.
Q Dr. Leavy -- Dr. Levy, do you think that the FDA, in concluding, as they did in 2014, that a cogent biological mechanism by which talc might lead to ovarian cancer is lacking, do you think they were wrong at that time?
A I would -- I -- I would say that they were incomplete at that time. And, in fact, you know, one of the --
If we -- if we look at page 5 in the

Page 205

one, two -- third full paragraph beginning with "while there exists," where the FDA does agree about the -- that it's plausible that perineal talc and other particulates reach the endometrial cavity and -- and associated organs and may elicit a foreign-body-type reaction and inflammatory response that in some exposed women may progress to epithelial cancers. What they do state, "However, there has been no conclusive evidence to support causality."
So I would suggest that this paragraph is in support of the biologically plausible mechanism that I included in the report and that -- and, as we've been discussing, I haven't -- we -- we've not been discussing a causal or a formal causal evaluation.
Q What information did you rely on, Doctor, in reaching the conclusion that there is a biological mechanism that the FDA did not?
MS. O'DELL:
Object to the form. Misstates his testimony.
A I'm stating that the -- as we discussed, as we've been discussing today, the --

Page 206

the response to talc -- the response to talc exposure as an inflammatory response is supported by a number of studies, including the NTP study, which, although the FDA had some concerns with, the FDA also made statements regarding the exposure to talc and other particulates having an inflammatory response and that some exposed women's may have progressed to epithelial cancers.

So, again, they're -- I think they -- they're in agreement there. So even the concerns with the study withstanding, there's -- there's -- there's -- I still -- I still think the FDA report is in support of the mechanism that we've been discussing.

MS. BROWN:

Q The FDA concludes that a cogent biological mechanism by which talc might lead to ovarian cancer is lacking, do they not?

MS. O'DELL:

Objection to form. Asked and answered.

A But I would al- -- I would say the FDA contr- -- perhaps contradicts itself later in the same document, stating that there is both an

Page 207

inflammatory response and that in some exposed women they may progress to epithelial cancer.

MS. BROWN:

Q Other than the Woodruff article, Doctor, are you aware of any other study in animals that shows inflammation leading to cancer?

MS. O'DELL:

Objection to form. Other than those he's mentioned?

A Yeah. I -- I would have to -- that would -- that would require review of the literature to -- to speak generally to animal studies and inflammation leading to cancer.

MS. BROWN:

Q Let me rephrase.

In terms of your opinion here that talc causes chronic inflammation that causes ovarian cancer, you identified the Hamilton study, the NTP study, and the Woodruff study as animal studies that support that view. True?

A I identified those studies as supportive of my -- of my opinion, yes.

Q Are you aware of any additional animal

Page 208

studies on which you're relying?

A Not -- not for the contents of the report. Not that I'm aware of. I think we've -- we've already discussed some of the other references contained in the report below and -- or at least by mention and Gates.

(DEPOSITION EXHIBIT NUMBER 17 WAS MARKED FOR IDENTIFICATION.)

MS. BROWN:

Q I'm gonna mark as Exhibit 17 to your deposition the Buz'Zard study that you mentioned a moment ago. Do you recall that?

A Yes.

Q Do you rely on the Buz'Zard study in supporting your view that chronic inflammation from talcum powder use can cause ovarian cancer?

MS. O'DELL:

17?

MS. BROWN:

Yes.

A Sorry. Can you restate your question? It wasn't...

MS. BROWN:

Q Do you rely on what we've marked as

Page 209

Exhibit 17, the Buz'Zard study, to support your view that talcum powder causes chronic inflammation that leads to ovarian cancer?

MS. O'DELL:

Object to the form.

A As we've discussed, not singularly, but the -- as part -- as part of a complete picture of talc causing reactive oxygen species generation and other inflammatory responses, certainly this is a study that supports that opinion.

MS. BROWN:

Q Did you consider the type of cells that were evaluated in the Buz'Zard study?

MS. O'DELL:

Objection to form. Vague.

A Certainly in terms of the overall experimental design.

MS. BROWN:

Q Did those -- were those normal human ovarian cells?

A The -- the author has labeled them as normal human ovarian cells. But the -- you know, one of the key characteristics and similar to our

Page 210

comments on -- on animal systems is all -- all in vitro or in vivo studies that are using cell lines or animals have limitations. And in this case, you know, cell lines are particularly notorious in research in general for their -- for -- having to use care in extending findings to, you know, broad mechanisms in a -- in a complex organism or in the human body.
Q Sure.
What you're -- what you're saying is you've got to be careful taking the findings from one cell study and extrapolating that to humans. Fair?
MS. O'DELL:
Object to the form.
A The -- I think you have to be careful in evaluating each study in using the relevant components of that study and observations in that study as part of an overall mechanism and whether it's supportive or refutes such a mechanism.
So --
MS. BROWN:
Q Did -- did you exercise that care here as it relates to the Buz'Zard study?

Page 211

A So the Buz'Zard study, you know, primarily, as -- as referenced, was to illustrate a study that showed an increase in reactive oxygen species generation, and that's the -- the primary purpose, or I should say primary observation on the -- from this.
Now, certainly, the study contained more observations than that and certainly had some -- you know, a number of other components.
Q How does the Buz'Zard study support your view that talcum powder causes chronic inflammation that causes ovarian cancer?
A So the Buz'Zard study supports the view that exposure to talcum powder causes an inflammatory response.
Q And that inflammatory response you saw in the Buz'Zard study does not increase with increasing doses of talcum powder. Correct?
A I have to review. I believe that -- I believe their figures suggest --
You know, are you referring specifically to their reaction -- reactive oxygen specie generation?
Q Correct.

Page 212

MS. O'DELL:
Figure 3.
A Figure 3?
The one interesting observation in these two figures, both Figure 3A and Figure 3B, being the percentage of reactive oxygen specie generation in two different cell types, one in -- one in Panel A and one in Panel B, is -- what I did not see included, if I --
And I'm reading to see if I recall correctly.
-- was a -- the -- the cell viability assay that they use for normalization has a -- somewhat of a limitation in that it -- it doesn't measure cell senescence. It only measures cell death. And, so, they -- not to dis- -- not that I disagree with your observation that it did not show the sig- -- significant increase, but there is the possibility that the reason that you see an actual decrease in the RS generation at the higher doses of talc is that cells have gone senescent and are essentially no longer responding to that increased dose.
So I think there's at least two

Page 213

different ways to interpret some of these results. But I don't disagree with your observations regarding Figure 3.
MS. BROWN:
Q This study was conducted in a nutritional lab, not a cancer lab. True?
A I'm -- I'm not aware of the type of laboratory or even the...
Q And the study was -- the purpose of the study was to assess whether there was a certain effect of pine bark supplement? Is that right?
MS. O'DELL:
Objection to form.
A They were looking at the -- the effect of a proprietary -- as stated by the authors, a proprietary mixture of water soluble bioflavonoids extracted from French maritime pine bark.
MS. BROWN:
Q Uh-huh.
And did you investigate whether the ovarian cells that they used here were genetically altered?
A No, I did not investigate that.

Page 214

Q Did you --
I'm sorry. Were you done?
A No. I would say it's fair -- it's fair to say that, you know, that the -- whether they're genetically altered or not, the -- the -- you know, the same potential limitations as far as extrapolation to the human system would apply for any signs.
But, again, the purpose of the Buz'Zard study, as -- as referenced in the report, was to indicate that there are studies that have shown an increase in reactive oxygen specie generation under exposure to -- to talc. And I think the study is reasonably clear on that increase relative to control.
Q Except what this study showed, Doctor, is the more talc you give, the decrease from baseline in the reactive oxygen species. Correct?
MS. O'DELL:
Object to the form. Asked and answered. Misstates the testimony.
MS. BROWN:
Q Take a look at Figure 3; right, Doctor?

Page 215

A No. I agree. But, as stated, and an important clarification is whether that decrease is significant relative to the biology is -- is unknown.
Q Right.
This study certainly does not conclusively show that the more talc you give, the more ROS is generated. Correct?
MS. O'DELL:
Object to the form.
A In these particular cell lines under these conditions, the -- the study certainly did not draw that conclusion.
MS. BROWN:
Q In fact, what this study shows is the more talc you give, the less of -- of ROS generation you have. Doesn't it?
MS. O'DELL:
Object to the form.
A I think importantly in this study, the time dependency for each of the doses is more important at the doses rather than comparing dose to dose.
MS. BROWN:

Page 216

Q My question was, Doctor, what this study shows is the more talc you give, the less ROS generation there is. True?
MS. O'DELL:
Objection to form.
A Again, under -- under the conditions of this particular study.
MS. BROWN:
Q Do you think the Buz'Zard study is scientifically reliable?
A I have no basis to -- to suggest that it's -- that it's not reliable.
Q Do you think that --
A But I think there -- it does -- if there is a -- as we discussed earlier, an importance to not overgeneralize conclusions or lack of conclusions as, you know, outside of the system under study.
Q If -- I want you to assume that the Buz'Zard study used genetically altered ovarian cells that did not have the p53 protein. Would that affect your analysis of Buz'Zard?
MS. O'DELL:
Object to the form.

Page 217

A Well, that's -- that's an impossible question. Like you can't have --
Well, you can't call a cell type a normal ovarian cell and -- absent p53 protein. You're -- it'd be -- you're fundamentally changing the biology of the cell as it relates to ovarian cancer or cancer in general.
MS. BROWN:
Q Because p53 is something that you have in your genes that prevents against ovarian cancer. True?
MS. O'DELL:
Objection.
A So p5- -- p53 is a well-known, often mutated gene in a number of human cancers.
MS. BROWN:
Q And, so, if the ovarian cells that were studied in Buz'Zard did not have p53, it will call into question the study. Fair?
MS. O'DELL:
Object to the form.
A It would be difficult to answer. From the perspective of the presence or absence of -- of p53 having an effect on the ability of a

Page 218

cell to generate reactive oxygen species under -- under exposure to a substance like talcum powder would need to be tested directly.
MS. BROWN:
Q Fair to say, in your mind, a cell missing p53 is not a normal human ovarian cell. True?
A That is true.
(DEPOSITION EXHIBIT NUMBER 18 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q Handing you what we've marked as Exhibit 18 to your deposition, it's a review article titled "Perineal Talc Use and Ovarian Cancer," by Ross Penninkilampi.
Do you see that?
A I do.
Q This is an article that you cited in your report; correct?
A Correct.
Q Does this article support your view that there is a biolo -- in part --
Strike that.
Does this article, in part, support

Page 219

your opinion in this case that there is a biologically plausible mechanism by which talcum powder can cause ovarian cancer which can cause --
Strike that. Gonna do it again.
Does this article support your view, in part, that talcum powder can cause chronic inflammation that can cause ovarian cancer?
A This is an article I considered in the -- in the overall review and, in the conclusions of this article, found a -- an association between perineal talc use and ovarian cancer, according to the authors.
So it was supportive of the proposed mechanism but was, again, in part.
Q And, on page 13 and 14 of your report, you, in fact, reference the Penninkilampi study and some of its conclusions; correct?
A Correct. On the -- on the bottom of page 13, yes.
Q And what was the purpose of including this description of Penninkilampi in your expert report, Doctor?
A Just to be sure to be -- to include

Page 220

available literature and, in this case, review a meta-analysis of some reasonably large-scale studies to try to bring the proposed biologically plausible mechanism and include the -- the available epidemiological information for those, such as the Penninkilampi and Eslick paper we're discussing.
Q What methodology did you employ in terms of reviewing the Penninkilampi findings as it relates to the question you addressed in your report?
MS. O'DELL:
Object to the form.
A I -- I used the same methodology for the other studies as a review of the paper and its -- and its methods and conclusions.
MS. BROWN:
Q Do you believe this review, systematic review and meta-analysis, provides evidence that there's a biologically plausible mechanism by which talc can cause ovarian cancer?
A Yes. It provided -- it shows an association between talc use and ovarian cancer. I don't -- I don't believe this particular study

Page 221

goes on to specifically elucidate causation, but it certainly shows the association.
Q Well, the study specifically says that causation cannot be found, based on the results. Right?
MS. O'DELL:
Objection to form.
MS. BROWN:
Q If you look at page 42, Doctor, the very end of that first paragraph, "A certain causal link between talc use and ovarian cancer has not been established."
Do you see that?
MS. O'DELL:
Where are you? Page 42. Where are you reading, please?
MS. BROWN:
Page 42, the end of the first paragraph.
A Yes, I see that.
MS. BROWN:
Q Do you agree with that statement, Doctor, that a causal link between talc use and ovarian cancer has not yet been established?

Page 222

MS. O'DELL:
Objection.
A No, I wouldn't. But, again, my review of this was to tie the biologically plausible mechanism to, you know, human observation, not provide a evaluation of the -- of the causal link.
And I think the -- I would suspect that the --
I'm also not aware of a study that has been able to -- or a -- or a -- what would be necessary --
I'm not aware of a study that has been able to provide all of the recognized and established methodology for causation and have that applied in -- in talc.
MS. BROWN:
Q You're not aware of any study in the talc epidemiology that has concluded that talcum powder causes ovarian cancer; correct?
MS. O'DELL:
Objection to form.
A I'm aware of a number of studies that have shown a strong correlation between the two.

Page 223

But I would have to defer to the epidemiology expert witnesses as to their opinion on causation.
MS. BROWN:
Q One of the things you told us that you reviewed in connection with your opinion was the talc epidemiology. Is that right?
A That's right.
Q Did you conduct a review of all of the available epidemiology on talcum powder use and ovarian cancer?
A I certainly tried to review it as comprehensively as -- as possible.
Q And, in connection with that review, you'll agree there is not a single study that concludes there is a causal association between talcum powder use and ovarian cancer; correct?
MS. O'DELL:
Objection to form.
A So I would -- I would -- interestingly, there -- it's -- it becomes a -- as more -- as more and more information has become available over the last few years, that becomes a more and more difficult bar to meet, simply because, to

Page 224

examine that comprehensively, when you consider the etiology of a disease and the latency periods that have been observed in ovarian cancer in general and the meta review by both this earlier paper by Penninkilampi and then their subsequent later work, you have a challenge of a -- in a cohort study, a disease that is somewhat rare, coupled with a exposure and latency period that's been, in the -- in the limited number of studies that have looked at this, appears to be quite long, and then when you couple in the -- the ethical concerns of actually performing a trial, where it becomes a very difficult causation bar to reach.
And, so, instead, we rely on the case -- the available case-control data and then systematic and meta-analysis reviews such as some of the epidemiologists have performed to make assessments into the likelihood that -- and the strength of the association between talc use and ovarian cancer.
Q Are you intending to provide an opinion on the strength of the association between talc use and ovarian cancer as evidenced in the

Page 225

epidemiology?
MS. O'DELL:
Object to the form.
A No. My -- my opinions are limited to the biologically plausible mechanism and then examining whether that biologically plausible mechanism presented is supported by observations in -- in available human studies.
MS. BROWN:
Q And when you say your opinion is limited to a biological plausible mechanism, are you talking of the theoretical concept or are you talking about in the context of women using talcum powder perineally?
A In the context --
MS. O'DELL:
Object to the form.
THE WITNESS:
Sorry.
MS. O'DELL:
Excuse me.
A In the -- in the context of women using talcum powder perineally specifically, and then -- and then certainly also the -- some of

Page 226

the fundamental aspects of that mechanism may apply to other exposures as well.
MS. BROWN:
Q Like what?
A Well, the -- the other exposure we've been discussing, in -- in that some of the studies looked at inhalation exposure, et cetera.
But the primary review and the primary opinion is based on the perineal use of talcum powder and that exposure that, as -- as we discussed earlier, has a -- certainly a strong association with perineal use and an exposure -- exposure in the ovaries.
Q Your opinion is that if a woman uses talcum powder perineally, there is a biologically plausible mechanism by which enough talcum powder can migrate from outside of her vagina to her ovary to cause chronic inflammation that can lead to ovarian cancer?
MS. O'DELL:
Object to the form.
A So I'd say that the first part of your question is well established and included in the statements from FDA and others that that

Page 227

migration does occur.
And then the next step in the -- in the mechanism is that that causes inflammation which, again, as we've discussed, in a number of studies, that the inflammation occurs and then, in these human studies, in their systematic review, that there is a clear association or a -- a observed association between perineal use of talc and the detection of ovarian cancer at some point in the -- in the women's lives and, in the case of the Penninkilampi, with a relationship to the number of lifetime applications.
So considering those things together, yes, there is a biologically plausible mechanism for perineal talc use through to ovarian cancer.
MS. BROWN:
Q Have you -- is -- is your opinion that there's a biologically plausible mechanism dependent on a particular number of years of perineal use?
MS. O'DELL:
Objection to form.
A The -- so the -- as we just discussed, there's no -- I can't point to a formal clinical

Page 228

trial that would examine that in a well-powered fashion to answer that question directly. And, certainly, as of today, there would be some significant ethical concerns with that design.
So, instead, we rely on the cohort and case-control studies that are available. And those, again, studies are supporting an association between talc use and ovarian cancer.
MS. BROWN:
Q Right. But I'm talking about for your opinion that it's biologically plausible for perineal use of talc to cause ovarian cancer, have you made a determination, in your mind, of how long that perineal use has to take place for?
MS. O'DELL:
Object to the form.
A I wasn't asked to provide -- to provide that opinion on -- and it -- on that length or exposure or duration.
Again, it was -- the focus was on the biologically plausible mechanism that if you have a single exposure and that -- that that single exposure through to any other may be sufficient to trigger that mechanism.

Page 229

MS. BROWN:
Q That's helpful, Doctor.
So, as I understand your opinion, your piece of the puzzle here was to look at whether one single application of talcum powder to the perineum could lead to chronic inflammation that could cause ovarian cancer.
MS. O'DELL:
Objection.
MS. BROWN:
Q Correct?
A No, no.
MS. O'DELL:
Object to the form of the question.
A No. That's not my -- my statement.
My statement was that, based on the evidence available, that there's a biologically plausible mechanism for the -- for the cellular changes that -- that is independent of the exposure.
MS. BROWN:
Q You've made a determin- --
A But certainly a single exposure would be the physically minimum number. And I

Page 230

believe -- I think we --
Q That's what I want to understand. And how you -- how you make this biological plausibility determination is to evaluate a single exposure? Is that right?
MS. O'DELL:
Object to the form.
A No.
MS. O'DELL:
Misstates his testimony.
A That's -- that's not what I'm stating.
My -- my statement is that the -- the biologically plausible mechanism is a mechanism that is independent of the exposure and that, as part of the description of that mechanism and the evaluation of the studies supporting that mechanism through an inflammatory response, the question of exposure, number, and duration, length of time, et cetera, would be a separate evaluation.
MS. BROWN:
Q Is your opinion that talcum powder products cause chronic inflammation that cause ovarian cancer limited to perineal use, or have

Page 231

you also evaluated body use of talcum powder products?
MS. O'DELL:
Object to the form.
A My -- my focus was on the perineal use, and that's where the majority of the studies have -- have examined. So the focus was on perineal use of talcum powder.
MS. BROWN:
Q And in conducting that evaluation, the results of which are contained in your report, you did not endeavor to quantify how much talcum powder used perineally could possibly migrate to the ovaries; is that right?
MS. O'DELL:
Object to the form. Asked and answered maybe ten times already today.
But you may answer the question.
A Yeah. I -- I wasn't asked to -- to provide that opinion or attempt that quantitation.
MS. BROWN:
Q So when you conduct your analysis of whether something can biologically cause an

Page 232

effect, it doesn't matter at all how much of the product is used?
MS. O'DELL:
Objection.
MS. BROWN:
Q Do you see what I'm struggling with? Can you help me understand? If I'm trying to figure out does X cause Y, it sounds like what you're saying is it doesn't matter how much X you have.
MS. O'DELL:
Objection to form.
A So we're -- we're talking about mech- -- so mechanistic action --
MS. BROWN:
Q Okay.
A -- which means the -- you set aside the "how much." And the question is, from -- on a molecular level, can the presence of a particular compound in a particular location cause a biological effect. And, so, that is the primary focus of the opinion in the -- in the paper or -- sorry -- in my report.
And then extending that to how much,

Page 233

how long, and the dur- -- and then the intensity or duration of the biological effect, again, is a separate -- would be a separate discussion or separate study.
So, again, to clarify, the focus had been on that -- some of the fundamental mechanisms, talc -- a talcum powder exposure to an inflammatory response to the inflammatory response causing cancer.
Again, the -- I would refer to and defer to the other experts in epidemiology regarding their opinions on the validity of the asso- -- validity and strength of the associations, again, from a formal epidemiology perspective.
My review of those studies has ind- -- has relied on their conclusions, and, then, in my own review of their -- of their methodology showing a increasing association, that is the bookends of my -- of the mechanism I proposed.
So what this study is looking at is perineal use of talc, getting cancer.
The -- what I've proposed is in the middle. But this, again, the epidemiology

Page 234

studies are asking how many times, what, and where, but there's been no evaluation that I'm aware of that looks at exactly how the talc was applied, when and where. Instead, it was asked number of lifetime applications, duration of use, and examining latency period.

And when I examine that information from the perspective of that biological mechanism, I, you know, notice some parallels in between latency period averaging roughly twenty years, which -- which mimics somewhat what's observed in the asbestos field as far as, you know, lung effect latency.

And then that continues into the constituent -- or the other constituent components of some of the products, including testing into asbestos and some of the -- and heavy metal exposure, et cetera, that those are, again, supportive and offer a potential amplifying effect in that -- in that mechanism, given the nature of those other components.

Q What's the scientific support for the amplification effect you just described?

A Just that the presence of

Page 235

more -- the --

So if we extend beyond the opinion that talc, as a com- -- as a singular compound, causes inflammation and then also, based on the reviewed expert reports, find that testing of talc has been shown to contain asbestos or asbestos fibers, that the presence of now two potential insulting --

I'm making a hypothesis or making a statement that the -- you can have -- the more biologically active compounds you have in an exposure such as talc plus asbestos plus chromium and then plus a milieu of chemicals that are in fragrances may have an amplification effect on that exposure and as part of that overall biological mechanism.

Q Are you relying on a particular article or any published scientific support for the amplification argument?

MS. O'DELL:

Object to the form. He's answered the question.

A No. I -- I don't know of a study that is delineated. The -- it would be synthesizing

Page 236

that opinion from the observations of a couple of different studies, including the recent Saed paper that did look at the specific consumer product every -- you know, showing a -- if we do it by way of comparison, between the Buz'Zard paper and the recent Saed, seemingly a larger magnitude of reactive oxygen species generation. But, again, that is a -- extrapolating against two different studies.

Q Do you --

MS. O'DELL:

Excuse me. We've been going about an hour and 20 minutes, maybe a little more.

MS. BROWN:

I think a little less. But I'm gonna finish up. Then we'll take a quick break.

Q Does that work for you, Doctor?

I just want to finish Penninkilampi if we can.

MS. O'DELL:

How much more do you have to go?

MS. BROWN:

About five or ten minutes.

MS. O'DELL:

Page 237

If you need a break, we can break now. Or we can keep -- if you would like to wait five or ten minutes, that's fine. Whatever's best for you, Doctor.

THE WITNESS:

Yeah, if we could break now, that would be great.

VIDEOGRAPHER:

Going off the record. The time is 2:10 p.m.

(OFF THE RECORD.)

VIDEOGRAPHER:

We're back on the record. The time is 2:26 p.m.

MS. BROWN:

Q Welcome back, Doctor.

Before we took a break, we were discussing the Penninkilampi article. Do you remember that?

A I do.

Q And one of the things the authors of this very recent meta-analysis discussed is the potential mechanism of ovarian cancer. Correct?

And I'll direct your attention to the

Page 238

discussion that begins on page 45. In the second sentence, the authors conclude here that the mechanism by which perineal talc use may increase the risk of ovarian cancer is uncertain.
Do you see that?
A I see that sentence, yes.
Q And they go on to discuss the theory that talc could produce a chronic inflammatory response which could predispose to the development of ovarian cancer.
Do you see that?
A Yes.
Q Okay. And they go on to explain a little bit more about the theory. Do you see that?
MS. O'DELL:
Object to the form.
A Specifically the sentence beginning with "it is argued"?
MS. BROWN:
Q Uh-huh. "It is argued that cellular injury, oxidative stress, and local increase in inflammatory mediators such as cytokines, prostaglandins may be mutagenic and, hence,

Page 239

promote carcinogenesis."
Do you see that?
A I see that.
Q This sentence refers to chronic inflammation promoting cancer. Correct?
MS. O'DELL:
Object to the form.
A No. This -- this refers to that the presence of -- proposed that talc as a foreign -- that the presence of a foreign body would instigate a chronic inflammatory response. That's the statement in the paper.
MS. BROWN:
Q Is it your opinion that talcum powder can cause chronic inflammation that initiates cancer?
A It's -- so it is -- it is my opinion is, part of the mechanism, that talcum powder can have two effects related to inflammation. The first effect is an acute effect resulting in cellular damage, and that is supported by the study showing increase in reactive oxygen species related to talc.
The -- beyond that, the continued

Page 240

presence of the talc or a continued chronic immune response or chronic inflammatory response, again, either directly or indirectly related to the exposure, would help support a environment that would allow the cancer progression to occur.
So that is simply delineating those -- those two things as it relates to inflammation and talc exposure.
Q So you described two potential responses to talc right now. Correct?
MS. O'DELL:
Objection to form.
A At least two, yes.
MS. BROWN:
Q Okay. And one is an acute inflammatory response; correct?
A Yes.
Q And for that you point to the Saed data on reactive oxygen species; is that right?
MS. O'DELL:
Objection to form.
A That is one example, yes.
MS. BROWN:
Q Okay. Are there -- is there other

Page 241

scientific support for your opinion that talc can cause acute inflammation?
A So it's any of the similar studies to Saed. And I would have to double-check the references, but they would have -- you know, any of the --
MS. O'DELL:
Feel free to --
MS. BROWN:
Q Buz'Zard?
A So Buz'Zard would be one. Harper and Saed is -- is another.
Q In your --
A And so -- yeah. Yes, Buz'Zard and Lau and then -- yeah. So that would --
Q Okay. So for your opinion that talc causes an acute inflamm- -- inflammatory response, you rely on the cell studies done by Saed and Buz'Zard; correct?
MS. O'DELL:
Object to the form.
A Yes, among others.
MS. BROWN:
Q In your opinion, Doctor, does that

Page 242

acute inflammatory response resolve?
A I don't -- I don't have any evidence to suggest it resolves or not. The --
Again, getting back to the mechanism that has been -- that I've described and is supported by the literature we've been discussing is that there is a acute response as well as evidence for talc causing a more chronic inflammatory response. And so I've proposed a mechanism by which both of those can contribute to or enhance the development of cancer.
Q Can both of those inflammatory responses that you just described initiate cancer?
MS. O'DELL:
Object to the form. Asked and answered.
A They are certainly a component of that.
And so, again, to restate the mechanism, the acute inflammatory response or the -- the formation of reactive oxygen species has been known for decades to cause cellular damage, and then cellular damage can result in mutation of -- of DNA.

Page 243

And then when you also consider the full constituents of the products, the potential presence --
And this gets back to our earlier discussions about amplification.
Components such as chromium, which have a direct DNA-damaging effect, can also ampli- -- again, add to the level of cellular damage present.
And then the continued inflammatory response, whether it is a -- related to the initial acute response and a continuation of that or is a separate chronic inflammatory response would then support the environment necessary for the malignant transformation or the malignancy of the cancer to become what we -- what we would generally refer to as ovarian cancer.
Q In your opinion, the chronic inflammation promotes the cancer but does not initiate it?
MS. O'DELL:
Object to the form. Asked and answered.
A No. So I wouldn't -- I would say

Page 244

they're not -- I don't have evidence to -- to delineate those specifically, other than -- other than the supported mechanism that an acute response can cause cellular damage, and then a chronic response can cause cellular damage and be supportive of that continued -- that continued transformation.
So they are -- they -- those -- those two delineated immune responses can either work in -- in concert with each other, but there is no evidence to suggest that one is insufficient relative to the other in terms of progression of the disease.
And I think specific to the -- to the supported mechanism is that there -- I'm not making that distinction in the -- in the report.
MS. BROWN:
Q Right. In your report, you don't talk about acute versus chronic inflammation. Correct?
A That's correct. I don't delineate the two. Right.
Q But, here today, as we discuss in more detail your opinions, you're explaining that

Page 245

you're -- in your mind, you see two potential inflammatory responses from talc. Right?
MS. O'DELL:
Object to the form.
A I would disagree. I would say that I -- I -- based on the information and studies, the -- the review of other expert reports, that it presents a supported opinion that talc has an ability to cause an acute response as well as a chronic response.
And, so, then, today we are discussing using that data in support of the -- of the mechanism as to how those -- those two responses can work together or separately in the progression of ovarian cancer.
MS. BROWN:
Q At the time you wrote your report in November of 2018, were you of the view that talc can cause both acute and chronic inflammatory response?
A Yes. I mean, it was -- I was of the view it causes an inflammatory response. And then, as I continued to review information available, it became clear that the talc

Page 246

response, being an inflammatory response in totality, may have the ability to have those -- to -- to have two independent responses in tissues.
Q And, in your opinion, can both the acute inflammatory response and the chronic inflammatory response separately cause ovarian cancer?
A Under the -- the mechanism I've proposed, yes, that would be a -- a possibility that they could separately cause, given that they -- they're both inflammatory responses, they both cause cellular damage.
And in the case -- in this case, delineating the acute from chronic was more to clarify the cellular damage aspect, the transformative aspect of cancer from the -- the necessary tumor progression aspects of cancer to actually progress to disease.
Q In your opinion, Doctor, does talc always first cause an acute reaction and then a chronic reaction?
MS. O'DELL:
Object to the form.

Page 247

A I -- I -- I don't have evidence to -- to state that and would defer to some of the other expert witnesses, like Dr. Saed, for opinions on acute response versus chronic.
MS. BROWN:
Q In your opinion, though, you have at least delineated in your mind two different types of inflammatory responses. Correct?
MS. O'DELL:
Objection to form.
A I've -- I have described two mechanisms for inflammation that -- that both can -- are both supportive of the overall mechanism that we're discussing.
MS. BROWN:
Q And is it -- is there a length of time that differentiates an acute inflammatory response from a chronic inflammatory response?
A Certainly I would say there -- in my opinion, there would -- it would be a potential time dependency or a magnitude dependency to delineate an acute versus chronic response. But, again, for the purpose of the biological mechanism, separating them on those lines is not

Page 248

important.
Q So there is a length of time or an amount of exposure that would cause a chronic inflammation that is different from the length of time and the magnitude of exposure that will cause an acute inflammation?
MS. O'DELL:
Object to the form. Misstates his testimony.
A Yeah, no. Not -- that's not what I -- that's not what I've stated.
I've simply stated that if we -- if we look at the -- what is known about inflammation and the biological response to foreign bodies, you can have an initial acute response mediated by the immune system and mediated by some of the cellular damage that takes place, and then that same response may continue in a chronic form for some period of time and at some level of magnitude.
Now, certainly there is likely a dependency or, I should say, likely a relationship to the amount of exposure and the magnitude of that response.

Page 249

But, again, the -- the opinions here are specific to the mechanism and the initial elucidation of that response and, you know, not -- not on a quantitation of a -- a dose-response relation -- or a dose-response curve or relationship.
MS. BROWN:
Q Do you believe that every time a talc particle enters the human body, it produces a inflammatory response?
A All of the evidence would suggest yes.
Q Have you considered Heller's 1996 study on that score?
A I would have to --
On the score of inflammatory response?
Q Do you recall that Heller looked at benign ovarian tissue and identified the potential presence of talc?
A Sounds familiar.
Q I'll hand it to you.
(DEPOSITION EXHIBIT NUMBER 19 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:
Q Handing you, Doctor, what we've marked

Page 250

Heller's '96 article as Exhibit 19.
And what I want to ask you about is Heller's finding as it relates to no reaction to the talc particle. Did you consider that --
MS. O'DELL:
Object to the form.
MS. BROWN:
Q -- in forming your opinion here?
MS. O'DELL:
Excuse me. Object to the form.
MS. BROWN:
Q I'll direct you, Doctor.
On page 1508 of the Heller article, right above the comments section, "The investigators on this study concluded no evidence or response to talc, such as foreign body giant cell reactions or fibrosis in the tissue."
My question is whether, in your opinion, every time talc is -- enters the body, it necessarily produces an inflammatory response.
MS. O'DELL:
Object to the form.
A No. My opinion is that every time talc enters the body, that has the potential to cause

Page 251

an immune response.
MS. BROWN:
Q Have you made a determination about whether or not that always happens?
A I'll have --
MS. O'DELL:
Object to the form. It's vague.
A I'm not aware of any --
There -- there -- these -- none of the studies that have been reviewed have been designed to answer the question of "if ever."
MS. BROWN:
Q So, in your view, then, it's an open question about whether talc can be inside the body and not produce an inflammatory response.
MS. O'DELL:
Object.
MS. BROWN:
Q Is that fair?
MS. O'DELL:
Excuse me. Objection to form. Misstates his testimony.
A So my -- my -- my testimony regarding the mechanism is that there is a well-supported

Page 252

mechanism that talc causes inflammation and then inflammation has a role in ovarian cancer.
Extending that to circumstances where an exposure would not cause inflammation is -- is not germane to that -- to that mechanism and, in fact, again, not supported by literature to show that, you know, that a single exposure or some number of exposures are necessary or sufficient for a particular phenotype.
MS. BROWN:
Q So this Heller study purports to have found talc in ovarian tissue without an inflammatory response; right?
MS. O'DELL:
Object to the form.
A In looking at their --
Just one moment.
So this was a --
So is your -- is your question that the -- if the -- if the author showed talc being present in normal ovarian tissue?
Q Well, first my question is did you consider this article in connection with your opinions in the case?

Page 253

A I don't recall this article specifically, and I don't believe I cited it.
I guess there's -- no.
Q And then my second question, Doctor, is is it your opinion that every time the human body is exposed to particles of talc, it necessarily produces an inflammatory response that can either promote or initiate cancer of the ovaries?
MS. O'DELL:
Object to the form.
A No. My --
MS. O'DELL:
Vague.
A My comment was that the -- that any exposure to talc, particularly the perineal exposure to talc, has the potential to cause an inflammatory reaction.
I don't have any evidence that all of the studies that we've been reviewing are in support -- are in support of that mechanism, but I don't know of a study that perhaps has been able to draw a conclusion, from a similar size study, to show that you can get significant talc accumulation without an inflammatory response.

Page 254

MS. BROWN:
Q Do you think you need significant talc accumulation in the human body to cause or promote ovarian cancer?
MS. O'DELL:
Objection to form.
A I wasn't asked to -- to provide -- provide that opinion.
And, again, referring to the studies that have -- that were reviewed and included in the report, there is a relationship between lifetime exposure and an increased risk in the epidemiology reports.
But more detail on that in this discussion, I would defer to the epidemiology experts. But the -- there -- there does appear to be a -- more of a response based on more talc in the -- in the studies referenced.
MS. BROWN:
Q So on --
Do you have any reason to dispute the findings of Heller here of talc in the ovaries without a foreign body reaction?
MS. O'DELL:

Page 255

Objection.
A I guess my -- I have some -- I guess I have some concerns with some of the methodology as it relates to the detection of the...
MS. BROWN:
Q Do you think it's possible, Doctor, for talc to enter the body and -- and be completely inert and not cause any reaction?
MS. O'DELL:
Object to the form.
A So my -- the -- the mechanism I've proposed is -- is based -- you know, based on the literature, is that talc causes an inflammatory response and that inflammatory response is supportive of progression to ovarian cancer.
MS. BROWN:
Q Does that happen 100 percent of the time?
MS. O'DELL:
Object to the form. In terms of inflammatory response or in terms of cancer?
MS. BROWN:
Q If you don't understand the question, you'll let me know.

Page 256

A In -- in terms of cancer, the epidemiology would suggest -- or I would say the -- the evidence in the literature is -- does not allow that question to be answered, and the reason being is when you look at the latency of the disease and the progression of the disease and the challenges in detecting it, there just has not been enough time with the, perhaps, rigor of analysis that is undergoing now to make that assessment of is it 100 percent of the time or is it something less than 100 percent of the time.
I think, statistically speaking, there -- the only data that -- that is available for review is -- is what is contained in some of the meta-analysis and epidemiology studies showing a significant increased risk to ovarian cancer based on exposure to talc. And it would -- it would only be -- I think it would be inappropriate at this time to try to infer what percentage of time that would be indicative of for exposure.
Q Have the plaintiffs' lawyers shared with you expert reports from their expert pathologists who have looked at ovarian tissue of

Page 257

plaintiffs in this litigation, purported to find talc with no foreign body reaction?
MS. O'DELL:
Objection. There have been no case-specific pathology reports disclosed in the litigation we're here about today. And if there's something else you're talking about, you should be specific.
A The -- I don't recall a pathology report. I've seen expert reports from epidemiologists, OB-GYN and -- and some -- and other scientists. But I don't recall a specific pathology report.
MS. BROWN:
Q If the biologically plausible mechanism that you posit in your report is true, would you expect that the pathology slides of women with ovarian cancer who have used talc would evidence talcum powder with a foreign body reaction?
MS. O'DELL:
Object to the form. Incomplete hypothetical.
A That, I would have to ask how you're defining a foreign body reaction.

Page 258

MS. BROWN:
Q Well, would you expect to see some evidence of inflammation in the ovarian tissue of women who used talcum powder products?
MS. O'DELL:
Object to the form. Incomplete hypothetical.
A Overall, speaking to, as we were discussing earlier, the potential for that inflammatory response remains. But given the heterogeneity in individuals, their overall health, their natural variation in the levels of activities of antioxidants, et cetera, I -- I would state that I would expect a variety of magnitude of response to a foreign body like talc among the individuals exposed to it.
MS. BROWN:
Q You'd expect to see something; right?
MS. O'DELL:
Object to the form.
A No, not necessarily, because it -- it very much depends on the timing that's -- that is observed, how -- what methodology is used to detect the presence of talc or detect the

Page 259

presence of the inflammatory response, if it's, you know, done histopathologically, if it is based on a reactive oxygen species assay.
So given the -- speaking in general terms, I think it's just inappropriate to make a conclusion as to that, yes, you would always expect to see something.
I would -- again, to restate what was stated earlier, any -- any exposure has the potential to cause that inflammatory response, and then the time, scale, and magnitude of that response is going to vary by person. Therefore, I would expect there would be a variability in individuals exposed to talc.
MS. BROWN:
Q Uh-huh. Is your opinion related to all the different histologic types of epithelial ovarian cancer?
A My -- my opinion is not exclusive to any -- any one type. Certainly, the epithelial serous being the more common and most virulent type of cancers I think represents the most common.
From a mechanistic perspective, I

Page 260

mentioned some of the other subtypes and the common gene mutations that go along with them and as, again, supportive of the same mechanism. And I think, if anything, the -- the current data would suggest a -- a higher prevalence of a particular subtype of cancer but certainly not the -- the mechanism doesn't -- is not exclusive to any one type.
Q In your view, all types of epithelial ovarian cancer can be caused by inflammation?
A No. That's -- that's not my statement. I would say all types of ovarian cancer are supported by an inflammatory response but that, as from a causative perspective, that's not what the mechanism is provided as an opinion as to cause. It's more that the -- an inflammatory response plays a role in disease initiation and/or progression.
Q In your view, Dr. Levy, it is biologically plausible for inflammation to cause all types of epithelial ovarian cancer; true?
A Again, I'm not -- I've not been speaking to inflammation as a causative -- as a cause of ovarian cancer. It is a factor in --

Page 261

in -- in disease progression.
Q So when you conclude, as you do in your report, that talcum powder products cause chronic inflammation, you do not conclude that that chronic inflammation causes ovarian cancer?
MS. O'DELL:
Object to the form.
A I wasn't asked to provide a causation.
MS. BROWN:
Q Your opinion here is limited to the potential for talcum powder products to produce inflammation; correct?
MS. O'DELL:
Object to the form.
A No. My -- so my opinion is a -- is a supported plausible biological mechanism by which the exposure to talc can lead to ovarian cancer. And, in my opinion, as supported in the -- in the report, that is through an inflammatory response.
MS. BROWN:
Q I must be missing you, Doctor. So are you of the opinion that inflammation can cause ovarian cancer?
A I'm of the opinion that inflammation is

Page 262

a component of ovarian cancer.
Q Well, I'm not sure what you mean by that. Can inflammation cause ovarian cancer?
MS. O'DELL:
Object to the form. Asked and answered.
A I'm asked -- I suppose -- again, the opinion here is of a mechanistic opinion, not a causation. I would defer to some of the epidemiology experts to have opinions on causation.
MS. BROWN:
Q You don't have an opinion on whether or not inflammation can cause ovarian cancer?
MS. O'DELL:
Different question.
A Correct. That's a --
As we've been discussing, my opinions are that inflammation is a component of ovarian cancer and can be attributed to aspects, not exclusively, but contributing to aspects of its initiation and aspects of its progression. But I did not say that ovarian cancer is caused by inflammation.

Page 263

MS. BROWN:
Q And what scientific support do you have for your opinion that inflammation is a component of ovarian cancer and can be attributed to aspects of ovarian cancer, including its initiation?
A So, again, the synthesis of the -- of the papers we've been discussing, including Saed and others, showing the reactive oxygen species produced from talc. And, then, as far as inflammation and its role in cancer, there are -- and it's a fundamentally accepted aspect of cancer biology that's been around for -- for quite some time. And we mentioned earlier that there's a variety of review articles, including the ones we were comparing sentences to earlier today, that describe that in great detail.
Q It's not generally accepted, though, that ovarian cancer is caused by inflammation. Fair?
MS. O'DELL:
Object to the form.
A I think there's a number of studies that --

Page 264

Well, first, we're -- I want to be cautious with our use of the word "cause" and -- because that's, as we've been discussing, this is a -- it is -- it is not controversial that ovarian cancer -- inflammation plays a role in ovarian cancer and -- and, again, my opinion is not towards causation.
MS. BROWN:
Q Well, I mean, tumors themselves elicit inflammatory responses; right?
A What -- so what -- specifically, what are you referring to?
Q Well, you talk about tumor-activated macrophages in your report; right?
A Yes.
Q There is an inflammatory response that's produced by the tumor itself; correct?
A Yes. There are -- there -- there -- there are absolutely cancer progression markers that are associated with continued inflammation.
Q And that has nothing to do necessarily with the events that cause the cancer. Right?
MS. O'DELL:
Object to the form.

Page 265

A Well, so the -- we -- we would be going down a slightly different road. And if we're -- so cancer as a complex disorder, you know, begins with an initiating event. But there is -- there is absolutely tumor evolution from that initial event through the progression of the disease.
So to state that the -- in the initial inflammatory response to the tumor is -- is not causative to the continuation of the disease I think would be incorrect.
MS. BROWN:
Q The Penninkilampi authors -- to conclude our discussion here -- concluded that the paragraph you were looking at with the sentence "The potential mechanism by which genital talc is associated with an increased risk of ovarian cancer, hence, remains unclear," do you see that?
A Yes.
Q And this meta-analysis was published in January of 2018; correct?
A Correct.
Q And it is, in fact, cited in the

Page 266

majority of the plaintiff expert reports in this litigation. Did you see that?
MS. O'DELL:
Object to the form. If you know that. Don't speculate.
MS. BROWN:
Q That's why I asked "Did you see that?"
A So I didn't specifically look at if this was referenced. I -- I certainly referenced it. But I would also point out another important part of the -- of this same reference, a -- about halfway down the following paragraph, beginning with "If chronic inflammation due to ascending foreign bodies is indeed the mechanism by which talc use is associated with ovarian cancer risks, then these results fit the picture."
So I think the authors were both describing some things that remain unclear but also offering some comments that are supportive of our earlier discussions today on this mechanism.
Q And your opinion here today, Doctor, is limited to the potential mechanism; right?
MS. O'DELL:

Page 267

Object to the form.
A So my -- my opinion is -- is -- is regarding a biologically plausible mechanism. But, then -- and, in doing so, have reviewed some of these studies that we're discussing now.
MS. BROWN:
Q Good.
And, as it relates to that potential mechanism, these Penninkilampi authors conclude that the potential mechanism remains unclear. Right?
MS. O'DELL:
Objection to form.
A They -- the article makes a statement, "The potential mechanism by which genital talc is associated with an increased risk of ovarian cancer, hence, remains unclear."
However, as we've been discussing, they go on to state, "If chronic inflammation due to ascending foreign body is indeed the mechanism," then there -- the results in this paper are -- fit that model.
So I think they're making reason- -- making reasonable statements based on the

Page 268

available data that there is a biologically plausible mechanism surrounding and, indeed, in the previous paragraph at the end of it where they discuss use of -- or expression of cyclooxygenase 1 and 2 as well as the action of NSAIDs, again, supportive of -- somewhat supportive of the inflammatory model. But...
MS. BROWN:
Q Well, as it relates to the NSAIDs, Doctor, they point to the fact that the NSAID data is inconsistent, at best, as evidence supportive of their conclusions that the mechanism is unclear; right?
A No. They point to it as -- they actually try to clarify that the -- the seemingly contradictory data regarding the NSAID use can be explained by the relatively low expression of cyclooxygenase 1 and cyclooxygenase 2, which are the targets of most common NSAIDs.
Q What they say is that the use of nonsteroidal anti-inflammatory drugs, NSAIDs, is not inversely associated with the incidence of ovarian cancer as may be expected if the etiology was related to chronic inflammation. Right?

Page 269

MS. O'DELL:
Objection to form.
A Yes, that statement is made. But, importantly, it is incomplete without the next sentence, again, explaining that -- that apparent -- that apparent question.
So if the -- if NSAIDs are not effective in ovarian cancer and the -- and, in turn -- and if the observation is also made that ovarian cancer cells don't express cyclooxygenase 1 and 2, then they would not -- they would be nonresponsive to NSAIDs.
Q You state on page 12 of your report, Doctor, in the last paragraph, the second-to-last sentence that begins "moreover," that the effect of nonsteroidal anti-inflammatory drugs, NSAIDs, to reduce the risk of ovarian cancer provides additional support for what you're discussing here, which is that chronic inflammation plays a key role in the development of ovarian cancer.
Right?
A Correct.
Q And that is, in fact, the opposite of what the authors in Penninkilampi report as

Page 270

relates to NSAIDs; right?
MS. O'DELL:
Object to the form.
A Not -- not necessarily. So there's -- getting back to the -- the specific cells under question and the inflammatory response being examined. And, so, if we are lowering overall chronic inflammation through the use of an NSAID is -- is one question. A separate question is is a -- is a ovarian cancer cell responsive to NSAIDs. So they're two separate biological phenomenon.
And, in one case, if those cells are not expressing the cyclooxygenase 1 and 2, they'll be nonresponsive.
I would speculate that NSAID use in the rest of the body would still result in the expected effect due to, you know, the -- due to the inhibition of cyclooxygenase 1 and 2.
So I don't think they're necessarily in conflict with each other.
(DEPOSITION EXHIBIT NUMBER 20 WAS MARKED FOR IDENTIFICATION.)
MS. BROWN:

Page 271

Q Handing you what we've marked as Defense Exhibit 20 to your deposition, this is a paper by Merritt entitled "Talcum Powder Chronic Pelvic Inflammation and NSAIDs in Relation to the Risk of Epithelial Ovarian Cancer."
Do you see that?
A I do.
Q And, in fact, on page 12 of your report, you cite this Merritt article. Correct?
A Yes. Uh-huh.
Q And you cite it for the proposition that studies have found a relationship between pelvic inflammatory disease and ovarian cancer risk. Correct?
A Correct.
MS. O'DELL:
Object to the form.
MS. BROWN:
Q And you point to Merritt when you determine here as a finding of a relationship between pelvic inflammatory disease and ovarian cancer in support of your opinion that inflammation can cause ovarian cancer. True?
A I'd have to double-check that

Page 272

statement.
And then there was, I think, importantly, the Lin 2011 paper is also relevant.
Q Well, as it relates to the Merritt paper, this cite is wrong; right?
A I need a moment to --
Q Let's look at what Merritt actually found about pelvic inflammatory disease.
If you look --
MS. O'DELL:
If you need a moment --
Excuse me. I'm sorry. I didn't mean to interrupt you.
If you need a moment to refresh yourself, Dr. Levy, please do.
MS. BROWN:
Q Sure. And if you -- when you're ready, Doctor, I'll direct you to the second column on page 174, and I want to talk about the last paragraph there that begins "if inflammation."
A Page?
Q And I'll read it into the record while you orient yourself. It's page 174, right-hand column. Final paragraph states, "If inflammation

Page 273

plays a role in the etiology of ovarian cancer, then it would be expected that PID would be associated with increased risks of ovarian cancer. PID is not associated with elevated risk of ovarian tumors in our data, confirming several previous reports of no association with PID in studies of all subtypes of ovarian cancer."
Did I read that correctly?
A You did.
Q All right. So you cited this study for the proposition that studies have found a relationship between PID and ovarian cancer risk. Right?
A No. I said -- I cited -- I said studies have found a relationship, yes, between PID and ovarian cancer risk.
Q And, in fact, this study did not find a relationship between PID and ovarian cancer risk. Right?
A I think this study found a -- I'm just looking at the...
So -- I'm sorry. Would you ask your question again? This -- this study did not find your --

Page 274

Yes, I --
Q Sure. I just -- you cited this study for the proposition that it showed there was a relationship between pelvic inflammatory disease and ovarian cancer risk, but, in fact, the study showed the opposite. Correct?
A Well, to be clear on the wording, stated that the studies have found a relationship. I didn't indicate whether it was positive or negative.
But I think, importantly, the study also has an important paragraph that is probably more related to its inclusion, which is on the same page we were just on, 174, second full paragraph in the discussion.
Q One of the things on this page, Doctor --
MS. O'DELL:
Are you finished, Doctor?
A I think important to at least finish that thought.
That paragraph reads, "Focusing on talc use, we found that any use of perineal talc was associated with a small but significantly

Page 275

increased risk of ovarian cancer overall and specifically amongst the invasive and LNP serous tumors, although no clear dose response with increase in duration of use was identified. This finding is consistent with results of previous studies."
So in the case of the report and the biologically plausible mechanism that's been supported by these studies, these studies differentiating the process of pelvic inflammatory disease doesn't ex- -- doesn't exclude or refute the inflammatory role or the role inflammation may play in ovarian cancer.
Q What this study concludes is that, on balance, chronic inflammation does not play a major role in the development of ovarian cancer. Do you recall reviewing this in connection with your opinions in this case?
MS. O'DELL:
Object to the form. Misstates the exhibit.
MS. BROWN:
Counsel, I'll direct you to the last paragraph of the abstract on page 1 which reads,

Page 276

quote, "We conclude that, on balance, chronic inflammation does not play a major role in the development of ovarian cancer."
Q Do you see that, Doctor?
A I see that.
Q And what this study did was it endeavored to look into factors potentially associated with ovarian inflammation to see if it could support the theory that chronic inflammation plays a role in ovarian cancer; right?
MS. O'DELL:
Object to the form.
A I would need to -- this one limitation of this particular paper is that it is connecting inflammation as evidenced by pelvic inflammatory disease and assuming that that source and type of inflammation would be -- the fact that there's not a direct association between -- or an increased risk of ovarian cancer in the presence of pelvic inflammatory disease; therefore, inflammation must not play a role in ovarian cancer. So that is their conclusions.
MS. BROWN:

Page 277

Q Well, they looked at a bunch of different inflammatory conditions, didn't they? That was the focus of the study. The authors endeavored to look at a number of different pro-inflammatory factors and see if they influenced ovarian cancer. Do you recall reviewing that?
A I do. I think -- but, more importantly, when we look at the -- their specific statements that are surrounding the mechanism we're discussing today, which has to do with talc exposure and perineal talc use, I think their -- their statements in that sense, which have already been read, quite stand on their own.
So what this may indicate is a variety of types of inflammation do -- as present in other diseases, those individually do not or may not have a specific role in the progression of ovarian cancer.
But it does not -- again, it does not mean that ovarian inflammation at the site of talc exposure in the ovary can't have a role in the progression of disease where -- again, as we were discussing earlier, with inflammation, we're

Page 278

now connecting independent biological processes.
And I think you're -- I want to be sure we're clear and not drawing the use of the word "chronic inflammation" as meaning any inflammation and, therefore, if it's not associated with ovarian cancer, that inflammation can't have a role.
What we're speaking about in terms of this mechanism is inflammation caused by the perineal use of talcum powder in the ovary and the -- and the -- to explain that increased risk of ovarian cancer, what is a plausible mechanism.
Q The authors write, on page 74 -- 174, Doctor, second column, paragraph that begins with "It has been hypothesized," "It has been hypothesized that talc is linked to ovarian cancer development through inflammation," comma, "however evidence linking an inflammatory response with talc contamination of the ovaries is lacking."
Do you see that?
A I do.
Q And you disagree with that statement?
A I would -- I would suggest that a

Page 279

number of studies in the literature since the publication of this paper would -- would suggest that these conclusions may have been premature.
Q Do you think that, at the time this paper was published in 2008, that Merritt was accurately representing the data as it related to whether chronic inflammation could play a role in the development of ovarian cancer?
MS. O'DELL:
Object to the form.
A I would say that Merritt has an unresolved -- has a number of unresolved conclusions or partial conclusions in their paper, again, including the paragraph we've discussed where they comment on the talc use with an increased risk of ovarian cancer.
MS. BROWN:
Q Did you see the confidence interval on that finding, Doctor?
A I'd have to -- in --
Is this in this paper or in the number of the --
Q You reference the finding of an association between talc use and ovarian cancer a

Page 280

couple times, and that's a 1.17 relative risk that you're referring to. Is that right?
A Where is that?
Q I'm looking at -- in the abstract.
A Yes.
Q Right. And the confidence interval is 1.01 to 1.36. Right?
A Correct.
MS. O'DELL:
As to what finding?
MS. BROWN:
The one we're discussing.
Q And, Doctor, you know that one -- a confidence interval that begins with one is not statistically significant?
MS. O'DELL:
Object to the form.
MS. BROWN:
Q Did you know that?
MS. O'DELL:
Object to the form.
A Well, I would say the authors have stated in that abstract that it is statistically significant.

Page 281

MS. BROWN:
Q Sure, because it's 1.01. My question to you was do you know that a confidence interval that begins with one is not statistically significant?
This finding, Doctor, is barely statistically significant, isn't it?
MS. O'DELL:
Object to the form.
A Again -- again, it's a -- whether it's barely or whether it's tremendously statistically significant, it -- it's still a finding that I would say is in support of -- has been supported by other studies with similar relative risk numbers in the -- in the 1.2 range and above, as indicated.
MS. BROWN:
Q Finally, Doctor, at the very -- the very last sentence of this Merritt study we're discussing, on page 175, concludes, "However, experimental evidence that perineal talc use elicits an inflammatory response in the ovaries is lacking, and overall we conclude that chronic inflammation does not play a major role in the

Page 282

development of ovarian cancer."
And my question for you is what methodology did you employ to consider the findings of the Merritt paper in coming to your opinions contained in your report?
MS. O'DELL:
Object to the form.
A Again, as we've discussed earlier here today, the -- there's been no singular paper that had a specific role in -- in developing the biologically plausible mechanism contained in the report. And, so, this -- this paper, among many others, was -- was used.
MS. BROWN:
Q Right. But the findings of this paper is that talcum powder doesn't produce an inflammatory response that leads to cancer. Right?
A The -- the findings of this paper was that there's not an association of pelvic inflammatory disease and risk of ovar- -- of epithelial ovarian cancer.
Q They conclude that chronic inflammation doesn't play a role in the development of ovarian

Page 283

cancer; right?
A I think they've -- they've extended that observation regarding pelvic inflammatory disease to that conclusion.
But I think the studies that have come after this and other -- certainly other areas of review would suggest that those specific -- the wording of those specific statements may not be the most appropriate representation of the -- of the observations made in the -- in the Merritt paper.
Q So did you weight the Merritt paper less than some other papers that came after it? Or how did you --
What I'm trying to understand is your methodology for considering this paper, which seems to squarely conclude talc doesn't cause inflammation.
MS. O'DELL:
Object to the form.
A I'm not -- so I would -- I would disagree that -- this paper does not make those conclusions that talc does not cause inflammation. What they --

Page 284

Again, the observations in this paper are regarding chronic inflammation and its -- and its major role in the development of ovarian cancer; and, again, in this -- in the specific individuals that they've looked at, it's in regards to pelvic inflammatory disease.
And, so, as far as weighting that paper, it would be similar to other papers and other observations in the sense that it was -- that the mechanism that is supported by a wide variety of work considers a history of -- history of work in the talc, inflammation, and ovarian cancer fields both in basic research and epidemiology to come up -- to come to the conclusions and mechanisms that are proposed.
I don't -- I can't give you a specific weighting algorithm that was used on any -- any given paper.
MS. BROWN:
Q Did you consider Merritt's finding that evidence linking an inflammatory response with talc of the ovaries is lacking?
A I certainly considered their -- I considered their statements in the -- in the

Page 285

paper. And I would question the dichotomy of the -- of some of their statements regarding talc risk to cancer.
And the first question that would come to mind for this particular study is how they assessed talc-related inflammation in -- specifically in the ovary. I don't recall seeing how they made that assessment.
It, instead, seemed to me that their assessments were based on chronic inflammation as it related to other biological conditions and then extrapolating that to rate of ovarian cancer.
Q How do you think one should measure talc-related inflammation in the ovary?
MS. O'DELL:
Object to the form.
A Again, I wasn't asked to -- to provide that opinion. But I would reference the more recent Saed paper which -- and other molecular -- and other molecular studies and certainly defer to Dr. Saed as an expert witness to discuss appropriate measurements for talc-related inflammation in the -- in the ovary or ovarian

Page 286

cells.
MS. BROWN:
Q Have you spoken with Dr. Saed?
A I have not.
Q Have you requested any information from Dr. Saed?
A No, I have not.
Q Have you -- would you hold to the same opinion if you did not consider the work of Dr. Saed?
MS. O'DELL:
Objection to form. Vague.
A I -- the work of Dr. Saed is -- is a consideration among the wide variety of other literature contained in here. And Dr. Saed's work for in vitro analysis and the quantitation of specific reactive oxygen species is -- is a factor in and it is in support of the mechanism that I've proposed, which is that that mechanism does not rely on that study or any singular study for it to be valid.
MS. BROWN:
Q The mechanism you proposed, Doctor, is not yet generally accepted in the scientific

Page 287

community. Would you agree?
MS. O'DELL:
Object to the form.
A I wouldn't have a basis for that opinion. As -- as we talked about earlier, I haven't shared this mechanism to ask for that opinion.
MS. BROWN:
Q You haven't published the proposed mechanism that is the subject of your report. Is that right?
A That's right.
Q You haven't discussed the proposed mechanism that is the subject of your report with any of your colleagues at HudsonAlpha; correct?
A That's correct.
Q So whether or not the proposed mechanism that is the subject of your report would be accepted by your peers in the scientific community, that's not something you have yet evaluated; correct?
MS. O'DELL:
Object to the form.
A My -- I wasn't requested to provide a

Page 288

biologically plausible mechanism that was also peer-reviewed, and I would rely on or point you to a number of other expert reports, particularly in the epidemiology space from this case, where you'll find a great many parallels to -- to this case.
So I, instead, would state independently myself and other respected scientists have essentially developed the same opinions regarding mechanism in this -- in this particular space.
MS. BROWN:
Q Is there another plaintiffs' expert that you're aware of who holds the same opinion as you do on biological plausibility?
A Yes.
Q Who's that?
A Patricia Moorman, who is an epidemiologist whose report I had the opportunity to read yesterday.
Q Is there -- and -- and even though she's an epidemiologist, Dr. Moorman has a view on biological plausibility? Is that right?
MS. O'DELL:

Page 289

Object to the form.
A She has a view on --
In her report was a -- a view on mechanism -- on mechanism, which included the discussion of inflammatory response and its role in ovarian cancer, which parallels this report.
MS. BROWN:
Q Do you consider your proposed mechanism that is the subject of your report to be a novel concept in the scientific world?
MS. O'DELL:
Object to the form.
A Which part?
MS. BROWN:
Q Any part.
MS. O'DELL:
Object to the form.
A Again, I -- my -- the -- what was requested of me was not to develop a novel concept or even to describe an untested hypothesis. What was requested of me was to review the available literature and provide a biologically plausible mechanism for talc exposure to ovarian cancer. And, so, that's

Page 290

what -- that's what my report provides.
MS. BROWN:
Q Do you think there could be other biologically plausible mechanisms by which talcum powder would be associated with ovarian cancer?
A I haven't been asked to -- to make a review related to other biological mechanisms. I was asked to develop a biologically plausible mechanism. And upon review of the totality of the literature, this mechanism that -- that I've presented and provided in the report is, in my opinion, the correct mechanism.
Q Did you have complete autonomy in your task to develop a biologically plausible mechanism?
A Yes.
Q Were there any limitations on how you should go about developing this biologically plausible limita- -- mechanism?
MS. O'DELL:
Object to the form of the question to the degree that the question seeks --
MS. BROWN:
Form.

Page 291

MS. O'DELL:
No, no. If it goes to conversations with counsel, it is not form. It is attorney-client privilege and it's protected. Work product privilege is protected.
And, so, Dr. Levy --
MS. BROWN:
No. Counsel --
MS. O'DELL:
Excuse me. Excuse me. I'm directing my witness based on privilege, and I can do that.
To the degree that counsel is trying to seek the substance of discussions you had with counsel, those are protected, and I direct you not to answer.
To the degree there's something in your mind to respond that's not that, you may -- you may respond.
MS. BROWN:
Q And as -- as counsel well knows, because we've had this discussion earlier this week, the federal rules allow discovery of any material you relied on in forming your opinions.
And, so, my answer here -- my question

Page 292

for you here, Doctor, is, were -- was there any limitation placed on you that you relied on in trying to develop your biologically plausible mechanism?
MS. O'DELL:
What's allowed -- you're well aware of this, counsel, I know -- that what's discoverable is are there materials considered -- you can ask him that -- was there assumptions that he was asked to make -- that's discoverable -- and the compensation. Those are the three things. Not conversations between counsel and Dr. Levy. So --
MS. BROWN:
Counsel, you can instruct or we'll get the judge. We do not have time for your speeches. We're trying to finish up and let other people -- other people ask questions.
MS. O'DELL:
That's straight from the rules. You're well aware of that.
MS. BROWN:
So here's the question. If you want to instruct, we'll take a break and get the judge.

Page 293

Q Did you rely on any instruction from counsel regarding any limitations on how you were to attempt to develop your biologically plausible mechanism?
A No. I was -- I was not provided -- there were no --
I'm trying to make sure I answer to be correct. But my very simple and direct answer is the requests for the report were very succinct and were given without limitation.
Q Did you try to develop any mechanism that you rejected in connection with your report?
MS. O'DELL:
Object to the form. Vague.
A So I would best answer that by saying I did not develop an initial mechanism and, instead, began a literature review looking at the available literature in talcum powder inflammation in cancer, ovarian cancer, and then in related subjects, and then, through the course of that review, was able to synthesize the opinion that you have, that we've been discussing, in the report.
MS. BROWN:

Page 294

Q Do you consider the biologically plausible mechanism that is the subject of your report to be a hypothesis?
MS. O'DELL:
Object to the form. Asked and answered.
A No, no. In fact, it is not. And it's -- I think it's very fundamentally different than a hypothesis.
Because, again, to state, the activities that were undertaken was a review of the literature and then, based on that review, a mechanism that was biologically plausible. It is not hypothetical.
MS. BROWN:
Q Have you tested your biologically plausible mechanism?
MS. O'DELL:
Object to the form.
A Tested in the sense of --
So I would -- I would answer that as -- in -- in my opinion, I would suggest that this has been tested based on following the completion of the report and reading other similarly derived

Page 295

or similarly requested both literature, some of the publications that we've been discussing, as well as other expert reports that have, as we've just discussed, some parallel aspects.
So, from a formal scientific process, that is -- would not, I think, be considered a formal test. But from the perspective of this biologically plausible mechanism, other scientists undertaking similar methodology came up with similar results.
And, so, therefore, I would say that this report is -- continues to be supported by independent reviews and content.
MS. BROWN:
Q The other scientists that you just referenced are also paid experts for the plaintiffs; is that right?
MS. O'DELL:
Object to the form.
A I don't have knowledge of that specifically.
MS. BROWN:
Q Well, when you said other experts looking at the same thing came up with a similar

Page 296

mechanism, you mean other experts in this litigation?
MS. O'DELL:
Object to the form. Misstates his testimony.
A Other -- other material -- the materials that I was -- that I was provided.
MS. BROWN:
Q And those materials are in the form of other expert reports like yours; right?
MS. O'DELL:
Object to the form.
A They are.
MS. BROWN:
Q Are you aware of any nonlitigation expert that has arrived at the same biologically plausible proposed mechanism as you?
MS. O'DELL:
Object to the form.
A Well, I think -- yeah, in the sense -- in the sense of the number of publications we've been discussing and some of the more recent both reviews and -- and Saed's paper, I suppose, as we've been discussing, Dr. Saed has been funded

Page 297

for some of this work, but I would counter that with sponsorship of -- of studies that are subsequently peer-reviewed, I think are generally held to a scientific standard and rigor, and would suggest that his most recent work would fall under that and -- and, therefore, I would not consider that in the same realm as an expert report.
MS. BROWN:
Q Are you aware that the plaintiffs' lawyers funded Dr. Saed's studies?
A I am.
Q How do you know that?
MS. O'DELL:
Don't speculate. If you know it, testify to it.
A No. I'm thinking of --
That was disclosed during the discussion of the -- of the paper, and the question I asked and actually looked on the paper was to --
And this -- this was getting to my own opinion as to the appropriateness and the potential scientific rigor of the paper, and that

Page 298

was whether or not Dr. Saed disclosed that relationship, which is, of course, ethically a requirement for sponsored research. And, indeed, that sponsorship is made in the paper.
MS. BROWN:
Q Was it important to you --
Did you ask Dr. Saed about the funding for his paper?
A I did not. As we -- as we discussed, I haven't spoken with him.
Q Were you troubled by the fact that Dr. Saed's disclosure does not reference which side of the litigation he's working for?
MS. O'DELL:
Object to the form.
A Are you asking for my opinion on if it troubled me?
MS. BROWN:
Q Yeah.
A No.
Q It sounds like you did a little investigation and you were satisfied with the disclosure. Was that your testimony?
MS. O'DELL:

Page 299

Object to the form. He didn't use the word "investigation."
A I was satisfied seeing a disclosure made regarding funding, which, again, in the scientific climate I would -- or I would state simply I viewed the support of that study which subsequently goes out to peer review functionally equivalent to pharmaceutical support of a study involving a drug or a condition or a treatment.
The reality of the scientific space is -- is -- is funding sponsorship comes from a variety of cases. And in each institution, HudsonAlpha certainly, I'm positive Wayne State has a conflict of interest review board which Dr. Saed has to report to as far as the -- how he manages that potential conflict of interest. And given that he's at a reputable institution that I've actually done a fair amount of review work with over the years, being Wayne State, I'm reasonably -- or I would say I'm quite confident that his conflict of interest has been managed appropriately for the -- for the study that was reviewed.
MS. BROWN:

Page 300

Q Why is it important, in your mind, to disclose funding for a study?
A Well, it's, you know, ethical premise of -- of most scientific research or really all extramurally funded research that the funding sources are -- are always disclosed. And that's true for publication as well as presentation.
And, so, I think most -- most scientists, during presentation, will present a slide that shows their -- their funning support and all of its sources regard- -- whether it's public or private.
And then you'll notice in vast majority of publications, if they are grant supported, again, whether that grant is from a public or a private institution, those things are referenced. And, in fact, the U. S. Government has a requirement that grants be referenced in their -- in any publications that were supported by that money.
Q Do you have any critiques of either of Saed's papers?
A No. Not at this time.
Q Do you have any questions or anything

Page 301

that doesn't make sense to you, having reviewed the most recent one or the 2017 one?
A No. My focus, particularly on the most recent one, I actually found his molecular studies to be quite comprehensive and --
So there was -- there was no specific concerns that -- that I was able to identify. And, again, the -- in the -- in the version of the paper that -- that I -- that I was given.
Q And did you have any opportunity to check to see if you had an earlier version of that paper?
A Oh, I -- I'll be sure and do that at the next break.
Q Okay. Why don't we go ahead and take a break now. You'll take a look, if you wouldn't mind, to see if you have something other than what we've marked at the deposition.
I'm going to renew -- review my notes. I'm close to finishing, and then I'll hand it over to my colleague, Mr. Ferguson, who I think will have some questions for you as well. Okay, Doctor?
A Uh-huh.

Page 302

Q Thank you, Doctor.
VIDEOGRAPHER:
Going off the record. The time is 3:33 p m.
(OFF THE RECORD.)
VIDEOGRAPHER:
We're back on the record. The time is 3:48 p.m.
MS. BROWN:
Q Welcome back, Doctor.
Did you have an opportunity to take a look if you had an earlier version of Dr. Saed's manuscript?
A I did.
I did not.
Q Okay. And, so, during this deposition, you've referred from time to time to Dr. Saed's 2018 paper. Is that right?
A (Nods affirmatively.)
MS. O'DELL:
Object to the form. Excuse me.
MS. BROWN:
Q And you received that paper after you authored your report in this case; right?

Page 303

MS. O'DELL:
Object to the form.
A So I was referring --
Yes. I -- I -- the manuscript we were discussing was received after the completion of this. But, as we discussed earlier, the materials in the paper were presented in abstract form or long abstract form, and those are referenced in the report.
MS. BROWN:
Q And just to close the loop on one thing before I hand it over to my colleague, Mr. Ferguson, you had referenced an animal study by Woodruff earlier in the day. Do you remember that?
A Yes.
Q That paper doesn't have anything to do with talc; right?
MS. O'DELL:
Object to the form.
A Let me --
Yes, I -- you're -- the Woodruff 1979 paper is not the one I was -- I was wrong on the author. Give me a moment to...

Page 304

MS. BROWN:
Q And if that's not the one you were thinking of, Doctor, we can move on.
A I was thinking Henderson 1971.
Q And that's not an animal study; right?
A Maybe this -- this isn't the same one, then. I can certainly find it at the end if --
The -- it was a 1971 study involving a rat model that the major point and conclusion of the study was perhaps something that we've discussed that's been now well accepted that the talc can migrate, after exposure, into the ovarian tissue.
Q Are you aware of any study, Doctor, that talc on the exterior of a woman's vagina can migrate up the fallopian tubes to the ovary?
MS. O'DELL:
Object to the form.
A I am not aware of a study that tested that specifically.
MS. BROWN:
Q And did you consider, in connection with your opinions here, IARC's finding that the science regarding migration is, quote, "weak"?

Page 305

MS. O'DELL:
Object to the form.
A My -- my primary consideration of IARC was their classification of the talc and the -- and the fibrous talc, and I don't recall their conclusions of the migration science being weak.
And, in fact, it appears, as stated by the FDA, that the -- the migration question is -- is well resolved.
MS. BROWN:
Q Finally, Doctor, in connection with your opinions in this case, did you consider articles regarding whether stick lesions evidence inflammation?
A I'd have to review some of the literature for stick lesions specifically. But that --
Can you -- what are you referring to by stick lesions?
Q So do you understand that it's now believed, in terms of the -- where ovarian cancer begins, that it begins in the fallopian tubes, epithelial ovarian cancer?
A I certainly would agree that a -- the

Page 306

site of initiation, whether -- that it can begin in the fallopian tubes, yes, that there's been studies that have shown that evidence.
Q And some of the early lesions that have been found in the fallopian tubes are sometimes referred to as stick lesions. Are you familiar with that?
MS. O'DELL:
Object to the form.
A I'm not.
MS. BROWN:
So you haven't looked at any studies that have looked at stick lesions that have been removed from women to see if there was any evidence of inflammation?
MS. O'DELL:
Object to the form.
A That -- that -- I don't recall that as part of the review.
MS. BROWN:
Q Fair enough.
No further questions. I'll hand it over to Mr. Ferguson.
MR. FERGUSON:

Page 307

Thank you.
EXAMINATION
BY MR. FERGUSON:
Q Good afternoon, Dr. Levy. My -- my name is Ken Ferguson, and I represent Imerys in this matter. Do you know who Imerys is?
A Only that they're a mining company.
Q Okay. And I have some questions for you. I apologize for my voice. I've kind of had my allergies and then going into a cold, so it's kind of -- kind of stuffy. So I apologize.
If you have trouble hearing me or understanding me, let me know. Okay?
A Okay.
Q And -- and just -- I know you've been at this with Miss Brown for a little while, but if there's any question that you don't understand that I'm asking you, just let me know, and I'll restate it so I can make sure that we're communicating. Okay?
A Okay.
Q I want to talk to you, first of all, about a little bit more about what you do at HudsonAlpha Institute. So in the what's called

Page 308

the Genomic Services Laboratory --
Right? There's one of those at HudsonAlpha; right?
A There is.
Q Do you perform services there such as running clinical samples to report results to healthcare providers? Is that the kind of things you do?
A To be -- to be clear and to, importantly, differentiate the regulated lab versus the research laboratory, the Genomic Services Laboratory is a -- is a entity of HudsonAlpha that is responsible for research activities.
There is a separate wholly owned subsidiary of HudsonAlpha creatively named the Clinical Services Laboratory. So that laboratory is the laboratory that performs the testing. And to hopefully not provide a level of confusion, but the two laboratories coexist in the same space. And what this means is I have staff and equipment. Some is dedicated to clinical, some is dedicated to research, and some are shared between the two.

Page 309

So, in summary, the best way to consider the laboratory is that it's a clinical regulated laboratory that also performs research.
Any projects under that research umbrella are referred to as being in the Genomic Services Laboratory. Anything clinical is referred to the Clinical Services Laboratory. That lab has been CLIA-licensed now for going on five -- just past four years and has been CAP-accredited for three and a half.
Q So is it the Clinical Services Laboratory, then, that would perform services like running clinical samples to get results to healthcare providers?
A That's correct.
Q And -- and among those things that the Clinical Services Laboratory does, is that restricted to whole genome sequencing?
A Our currently -- the only publicly disclosed and validated test for the Clinical Services Laboratory is whole genome sequencing.
We have two other laboratory-developed tests, or commonly referred to as LDTs, that are run in a -- as a private assay for some clinical

Page 310

trials, so they're not publicly available and to date have not been publicly disclosed. They're protected under confidentiality agreement.

And the Clinical Services Laboratory this year will launch a number of other tests that we have publicly disclosed. Those include whole exome sequencing, an oncology panel known as the TruSight Tumor 170, which profiles 170 genes with -- that have been -- that have known involvement in cancer risk and progression, and as well as a 500 panel of similar form.

Q So let me talk to you a little bit about your prior position. You were at Vanderbilt University Medical Center; correct?

A Correct.

Q And you were an assistant professor? Is that correct?

A The titles I held there was research assistant professor and then assistant professor, and then I was a associate professor as an adjunct faculty for a number of years after joining HudsonAlpha. So I had to progress through a few of the academic ranks at Vanderbilt, but all of them in the professor

Page 311

realm.

Q As an assistant professor, were you appointed on a tenure track?

A Yes.

Q And do you know generally how many years after appointment as an assistant professor is a tenure decision at Vanderbilt typically made in that department?

A It varies from probably five to nine.

Q Did you ever achieve tenure at Vanderbilt?

A Actually, I was up for tenure the year that I moved to HudsonAlpha.

Q So --

A So, technically, I, which will sound odd, I was promoted to associate professor upon leaving.

Q Okay.

A In an adjunct role.

Q So were you turned down for tenure or --

A I was not. I never -- I -- the opportunity at HudsonAlpha predated the time that I would have gone up for tenure. I had a number

Page 312

of pre-reviews for tenure. There were no concerns with that progress. But, based on both funding as well as publication records, I wasn't overly concerned with that.

But the opportunity to be able to do -- and the scale of operations at HudsonAlpha was -- was too good to turn down, as far as remaining at Vanderbilt.

Q So you were neither granted tenure nor denied tenure. Is that fair to say?

A That's fair to say.

I think the best evidence for the relationship at Vanderbilt after my leaving was I continued as an adjunct faculty in the same department, again with change in title, for a number of years after joining HudsonAlpha. So it was a -- certainly, I wouldn't characterize it as a negative departure from the institution. And I still remain a collaborator with a number of colleagues there.

Q Do you have a copy of your report in front of you?

A I do.

Q Okay. What I'm gonna do is I'm gonna

Page 313

try to go through, probably in -- in order, portions of your report that I want to ask about and try to make sure I don't cover things that Miss Brown's already covered.

Can you look at page 5 of your report?

A Yes.

Q So there -- and I'm looking at number 2 on page 5, Acquired Somatic Gene Mutation.

Do you see that?

A I do.

Q And you say there that --

I'm skipping the sentences. If you need to go back, feel free.

-- "Biological and lifestyle exposures, such as viruses, obesity, hormones and chronic inflammation, are also known to result in cancer-causing mutations."

Right?

A I see that sentence.

Q Okay. Wouldn't you agree that the association between obesity and cancer risk is just that, an association and not a known cause-and-effect relationship?

MS. O'DELL:

Page 314

Object to the form.
A I would state that it is known that cancer rates increase in a number of unhealthy conditions, including obesity. But I am not aware of a -- of any studies that have illustrated a causal effect directly between obesity and cancer.
MR. FERGUSON:
Q And, specifically, isn't it true that there is no direct in vivo experimental evidence that obesity causes cancer-causing mutations?
A I would have to review the literature to -- before answering that question. But the relationship between obesity and cancer risk is -- is quite well established. And I think for us to discuss that in more detail, we'd have to start delving into some of the specifics around the physiological changes related to obesity and whether those specific physiological changes play a role in cancer.
Q And, just below that, the last sentence in that paragraph, you say, "These mechanisms may be direct, such as radiation directly damaging DNA, as well as indirect, such as an external

Page 315

agent causing a cellular -- cellular reaction or inflammatory response that then leads to DNA damage or mutation."
What cellular reactions are you referring to that result in DNA damage or mutation?
A So the presence of reactive -- so a few different things. Primarily, along the discussions for today, the presence of reactive oxygen species which can directly -- which are a cellular reaction that can then cause -- directly cause DNA damage.
There's protein oxidation effects that are similar to that, in the sense that you have a chemical change and a cellular component that results in a -- in a protein activity change, again leading to potential DNA damage.
And then you can have --
So those are two -- two examples of cellular reactions to that.
Q And -- and maybe you just explained it, but I wanted to make sure I'm clear. What is the mechanism by which an inflammatory response results in DNA damage?

Page 316

A It varies. So the -- the -- "inflammatory response" is a bit general. So depending on specific type of cellular recruitment and cellular damage through the release of cytokines, the release of oxidative damaging materials from cells like granulocytes, you know, or the -- even the cell's own production of reaction to -- reactive oxygen species, such as from the mitochondria, which is the most common sync -- or most common source of reactive oxygen species in the cell.
And, so, those are some examples of -- of that relationship between an inflammatory response and that cellular reaction.
Q Reactive oxygen species are not the same thing as inflammation; correct?
A I would say reactive oxygen species are a hallmark of inflammation.
Q But they're not the same thing.
MS. O'DELL:
Object to the form.
A The -- well, they are --
Again, reactive oxygen species are a component of inflammation. So they're -- the

Page 317

words are two -- two different definitions, but they are a component.
MR. FERGUSON:
Q Would you agree that reactive oxygen species are a normal part of cell physiology?
A Yes, absolutely.
Q And the major source of reactive oxygen species comes from inside the cell and is produced in mitochondria?
A A source, and depending on the site of the physiology. So a normal, healthy cell not under stress or injury would be -- then, yes, that's a true statement.
Under different physiological conditions, that statement may not be true.
Q Can you distinguish reactive oxygen species produced inside a cell from reactive oxygen species produced outside the cell?
A What do you mean? So by -- by "distinguish," you mean --
Q Can you tell the difference?
A I'm just thinking if there's a way to measure.
So you can measure the effects of

Page 318

exogenously introduced reactive oxygen species and then compare that to the measurement of endogenously produced reactive oxygen species.

But as far as determining the difference if the cellular integrity is not intact, I'm not aware of a method to do that.

Q Would you agree that generation of reactive oxygen species is an inevitable consequence of aging in aerobic organisms?

MS. O'DELL:

Object to the form.

A So reactive oxygen species are a -- are present at all stages of life. And aging, as a biological phenomenon, is probably one of the most variable phenomenon that exists.

And specific to reactive oxygen species, the diet, lifestyle, and genetics of that individual will drastically change that.

And a new area of research that my laboratory has been undertaking for a short time --

And, so, I don't have specific publications, and it's really not -- I promise it's not taking us too far afield.

Page 319

-- but is the concept of your annual age versus biological age. And my lab has some assays that are based on epigenetics as well as some metabolomic markers. And what we found -- now, in very, again, preliminary data -- that individuals will vary by plus or minus 15 years from physiological age to annual age based on, again, a number of lifestyle factors not important for this study.

But the point I'm making is the discussion about level of reactive oxygen species and its association with age is actually quite variable based on the long -- or based on the current physiological activity of that person.

Stated very simply, which is probably something we all know, the better shape you're in, the younger your physiology will appear. And you can actually modulate that quite quickly, meaning that a person who's 60 and has made poor lifestyle choices can actually gain back quite a bit of that physiological age quite quickly.

And so, again, to directly answer your question, a annual age-related conclusion regarding production of reactive oxygen species would be very difficult.

Page 320

MR. FERGUSON:

Q In your report, on this same page, you discuss the fact that, even if someone has a genetic mutation that predisposes them to cancer doesn't mean that he or she is certain to get cancer. Correct?

A That is correct.

Q So there is a -- a random component to the effects of known cancer-causing agents. Right?

MS. O'DELL:

Objection to form.

A There is a complicated relationship between genetics, environment, and expose -- or environment, including exposure and lifestyle, and the progression of cancer.

Perhaps the -- a summary analogy is the more predisposing mutations that an individual has, it's -- it's equivalent to their body is rolling the dice more often to collect a mutation sufficient to cause cancer than somebody who does not have the same genetic background.

And there's -- there's many, many lines of evidence. Probably the most prominent is BRCA1 and 2 mutation and the role it plays in increased risk of breast and ovarian cancer.

Page 321

MR. FERGUSON:

Q Wouldn't you agree that even the inherited susceptibility cannot entirely explain this random component of some people getting cancer when exposed and some people not?

MS. O'DELL:

Objection to form.

A DNA -- so that, it's very gene-dependent. So BRCA1 and 2 is the example given. That is correct, that if you have a BRCA1 and -- 1 or 2 mutation, you are not guaranteed to get cancer.

Corollary to that is if you do not have a BRCA1 and 2 mutation, your relative risk for canner does not change, meaning that you're at no less of a risk than somebody -- somebody else who doesn't have that mutation.

I should state that there are other genes. P53 is a good example that was mentioned earlier. If you carry a mutation in that gene, the probability that you'll get cancer, assuming

Page 322

you don't die from something else, is almost certain, meaning that it's in the mid to high 90 percents if you -- if you live until a late age.
MR. FERGUSON:
Q Further down this paragraph, you indicate that "An inherited gene mutation could instead make one more likely to develop cancer when exposed to certain cancer-causing substances."
Correct? That's your statement?
A Yes.
Q Can you provide any examples in which a woman with an inherited mutation in a particular gene has been demonstrated to have more sensitivity to developing ovarian cancer as a result of exposure to an environmental agent?
A Not for ovarian cancer specifically. I would need to review --
There is a -- I've seen report of a single gene related to ovarian cancer, which, again, I would have to do a bit of searching to be sure I'm naming the correct gene, but I -- where that has a much high- -- increased risk specific to ovarian cancer, but I do not recall

Page 323

if there was a measurement of any exogenous exposure risk that amplified that effect or not.
But I think the -- as a general premise, it is a -- well established in cancer biology that any mu- -- any mutation that results in a burden related to DNA repair, related to cell cycle control, you are more susceptible to cancer.
In one of our lines of research where we do have some publications, in pediatric cancer, I would simply point to in approximately 50 percent of adults who are survivors of childhood cancer will develop a second cancer event primarily because their -- the fact that they developed a childhood cancer generally means you are predisposed to that condition.
And -- and, as evidenced in the observations we've done in the analysis of thousands of patients in collaboration with St. Jude and the children's oncology group, we've identified now a ability to do genetic counseling in those individuals and predict with very high accuracy what their secondary cancer is likely to be.

Page 324

And the point of my mentioning this is to illustrate that an early predisposition to -- or a significant predisposition to cancer that results in a early cancer event, those individuals show a lifetime increase in risk of approximately -- they're -- they're approximately six times, depending on the disease, to 13 times more likely to get that -- to get a secondary disease.
So there clearly is a relationship to predisposition in -- in oncology -- or in rate of cancer event.
Q Okay. And I appreciate your response. But remember that my question was related to ovarian cancer, and -- and we went a little afield from ovarian cancer.
And I want to ask you another question in that regard. Can you provide any example in which a woman with an inherited mutation in a particular gene has been demonstrated to have more sensitivity to developing ovarian cancer as a result of exposure to talcum powder?
MS. O'DELL:
Object to the form.

Page 325

Answer the question.
A So the mechanism we proposed would be independent of -- of that predisposition. But I would have the opinion that an individual with any predisposition mutation, regardless of the gene but -- and -- in ovarian cancer, that they would be a more fragile individual as -- when it comes to this exposure under the mechanism that we've been discussing today.
MR. FERGUSON:
Q Okay. And what I'm looking for is some example or some literature in that regard.
A I would -- I would have to -- I would have to look --
Q Okay.
A -- to see.
Q So what you've told me is that's your opinion, but you don't have any references for it as you sit here?
MS. O'DELL:
Objection to form.
A So my -- what was -- I was requested to provide this biologically plausible mechanism, and part of that request was not necessarily

Page 326

include the influence on that mechanism that specific gene mutations or inherited risks may have within relation to ovarian cancer.
So I'd certainly be delighted to pause for a moment and take -- you know, and -- and work on that -- give you that -- see if I can give you that specific example.
MR. FERGUSON:
Q But you can't as you sit here?
A I cannot.
Q Okay. So let's look at -- further down on page 5, you have a section entitled "The Role of Genetics in Ovarian Cancer." Correct?
A Correct.
Q And I want to look at a reference that you -- you have cited. And let me mark this as an exhibit, please. I guess I can mark it.
(DEPOSITION EXHIBIT NUMBER 21 WAS MARKED FOR IDENTIFICATION.)
MR. FERGUSON:
Q Exhibit 21 is the Nunes article. Have you seen that?
A I have, yes.
Q Okay. So if we look at page 5, at top

Page 327

of the page, you indicate that ovarian cancer is the major cause of death from gynecologic disease and the second most common gynecologic malignancy worldwide; correct?
A Correct.
Q And then in your report you cite Nunes and Serpa, the article we've just marked as Exhibit 21, as well as Siegel and Torre; correct?
A Yes.
Q If we look at page 2 of the Nunes article, the exact same sentence appears on -- at the bottom of page 2 under the heading of "Ovarian Cancer, an Overview"; correct?
A Correct.
Q Right.
A That's correct.
Q Okay. And it's --
A It's not quite the same sentence, given that it's the same initial statement, not an identical sentence.
Q Very close to identical?
A Well, they -- they both -- they both introduce the same facts.
Q Okay. Then if we go down a little bit

Page 328

further and you have a sentence that starts "epithelial ovarian cancer." Correct?
MS. O'DELL:
On page 6 there?
MR. FERGUSON:
Yeah. I apologize. Yeah, it is.
A Yep.
MR. FERGUSON:
Q It's on page 6. It's the, I believe, the last sentence of the partial paragraph at the top of 6. See it?
A I do.
Q Okay. And you say, "Epithelial ovarian cancer (EOC) includes most malignant ovarian neoplasms" -- you cite Chan, 2006 -- "that can be classified based on morphologic and molecular genetic features into the following types: Serous" -- and, in parentheses, "(OSC) low and high grade); endometrioid (EC), clear cell, (OCCC), and mucinous (MC) carcinomas."
Correct?
A Correct.
Q Okay. And then if we look back at page 2 of Nunes, in the second sentence of the first

Page 329

paragraph under "Ovarian Cancer, an Overview," the nearly identical sentence appears there. Correct?
MS. O'DELL:
Object to the form.
A The two sentences stating the same fundamental facts regarding ovarian cancer and the histological types are -- yes, I agree.
MR. FERGUSON:
Q With almost the same wording.
MS. O'DELL:
Object to the form.
A They have similar wording.
MR. FERGUSON:
Q Remarkably similar; correct?
MS. O'DELL:
Object to the form.
A I wouldn't call it -- so they --
Again, we're stating fundamental basic facts around histological type and following a number of, again, factual observations for what the state of the art for genetic knowledge in -- in different genes and different proteins is as it relates to our understanding of -- of

Page 330

cancer with, again, appropriate reference for those -- for those studies.
MR. FERGUSON:
Q And then if we look at the following paragraphs, the first full paragraph there on page 6, in your report you have a sentence that starts "low grade OSC cases generally have genetic alterations" in a number of items you've listed; correct?
A Correct.
Q Okay. And that sentence ends with the words or "p13/Ras/Notch/FOXM1." Correct?
A Correct.
Q Okay. And then if we go back to Nunes, if you look at that same paragraph we've been talking about -- and those -- there's an introductory phrase that you don't have, and then it starts with "low grade OSC generally comprising." Slightly different wording, but you list the same types of receptors and the same types of items. Correct?
A Yes. That's providing a review of, again, the known associations between specific ovarian subtypes and their most commonly referred

Page 331

genetic information or genetic predis- -- sorry -- mutated genes. So I'm -- that's right.
Q Okay.
A They are -- they are similar in that both are, again, introducing factual information about the current knowledge in ovarian cancer in this literature, again pointing out that referencing the papers that they both came from, being the Nunes as well as the appropriate references.
Q Okay. And, then, the paragraph below that starts endo- -- "endometrioid carcinoma," paren, "(EC)." Correct?
A Correct.
Q If we look --
And then that goes all the way to the word "mucin-coding genes" with two citations; correct?
A Correct.
Q If we look at 2 and the top of page 3 in Nunes, there's a sentence that starts "EC." It does not spell out endometrioid carcinoma. Do you see that four lines from the top? I'm sorry. Four lines from the bottom --

Page 332

MS. O'DELL:
I'm sorry.
MR. FERGUSON:
Q -- on page 2.
A Yes.
MR. FERGUSON:
Sorry. Leigh, it's on page -- the bottom of page 2.
MS. O'DELL:
Oh, I'm there. When you said the top, I got --
MR. FERGUSON:
No worries. That's -- my mistake.
Q Okay. It says "EC subtypes," and then it goes to mucin-coding genes on the top of page 3. Correct?
A Correct.
Q Again, that paragraph is nearly identical to the one in your report. Correct?
MS. O'DELL:
Object to the form.
MR. FERGUSON:
Q Same word, same order, same citations; correct?

Page 333

MS. O'DELL:
Object to the form.
A So my -- my report is similar to the review article. It -- it's listing the subtypes of ovarian cancer and -- based on the Nunes paper, which is a 2018 publication, so a more current review. I'm, again, providing that referenced information about the -- the -- this observation.
Q You're citing the same references as Nunes; correct?
A Yes.
Q You cite the -- the various gene -- expression of gene in the same order they do, so --
Correct?
A Yes.
Q And is that just coincidental? That's just happened? You happened to have put this paragraph in the same order with the same notations as -- as Nunes?
MS. O'DELL:
Object to the form.
A Well, I'm listing the same information

Page 334

that's contained in the Nunes paper. And seeing as that -- this was a review of the literature with -- you know, based on the state of the art, the Nunes review is exactly that. And, again, I'm -- I'm repeating the information regarding the specific gene information as it relates to this -- this ovarian cancer risk and -- and -- and, again, appropriately citing the basic studies as Nunes did.
MR. FERGUSON:
Q With virtually the same wording?
A With similar wording, yes.
Q Let's look at page -- page 7.
MS. O'DELL:
His report?
MR. FERGUSON:
Q Yeah. I apologize. Your report.
We can set Nunes aside now.
You have a paragraph starts -- that starts "individuals can inherit mutations in BRCA1, BRCA2 or p53."
See it?
A Uh-huh.
Q And you say, "These defects allow

Page 335

additional mutations to accumulate in cells and lead to a higher probability of cells being cancerous."
Correct?
A Correct.
Q And you've indicated earlier in your report that cancer is caused by mutations. Correct?
A Correct.
Q And you say here that mutations in BRCA1, BRCA2 or p53 can result in the accumulation of additional mutations in cells. Correct?
MS. O'DELL:
Object to the form.
A Yeah. I made the statement that BRCA1, BRCA2 and p53, they can be inherited and then, in turn, positive for those gene mutations.
MR. FERGUSON:
Q Okay. Would you --
A So I guess if you could ask the question again to make sure I understand it.
Q Well, let me -- doesn't this paragraph mean, in your comments here, that BRCA1, BRCA2,

Page 336

or p53 mutations can be considered causes of cancer?
MS. O'DELL:
Object to the form.
A No. Not -- not specifically causal. I think the -- each of these -- as we've discussed, each of these genes, BRCA1 and BRCA2, or starting with BRCA1 and BRCA2, increase the probability of a -- of a person -- generally women -- getting breast or ovarian cancer but do not exclusively mean somebody with that mutation will get cancer.
So, with that knowledge, I would not consider BRCA1 and BRCA2 mutation alone sufficient to cause cancer. It increased the risk.
And, as we talked about, p53 is a bit more of a higher-risk gene, and the question as to whether or not it is possible for someone to have a -- what the rate of someone having a p53 mutation and not getting cancer, I believe, is currently unknown. But there, again, is a much higher probability of developing -- developing cancer.
MR. FERGUSON:

Page 337

Q And then the last line there of page 7, you say, "The lifetime risk for ovarian cancer is approximately 40 percent for BRCA1 carriers and 15 to 20 percent for BRCA2 carriers."
Correct?
A Correct. Based on -- based on the study that I referenced, yes.
Q Right.
And -- and the -- the -- if we look at the increased risk of 40 percent as compared to the risk of cancer in the -- of ovarian cancer in the general population, that's a 25-fold increase for BRCA1 and about a 7- or 8-fold increase for BRCA2; correct?
MS. O'DELL:
Object to the form.
A I -- I would have to -- to determine that. But I would say so. I'm certainly comfortable stating that the lifetime risk for ovarian cancer is approximately 40 percent. I'd have to verify your -- your math about that indicating a 25-fold increase.
MR. FERGUSON:
Q Do you know what the rate in the

Page 338

general population of ovarian cancer is?
A It's fairly low. If I -- thinking of the cohort studies that were reviewed as part of this, it was roughly a hundred to 200 cases per 30- to 40,000 women in those -- in those studies, so relatively low.
Q And if we go to the top of the next page, you say -- it's page 8 -- "Therefore, the presence of mutations in the BRCA genes do not guarantee that carriers will get cancer. The presence of these mutations increases a person's risk of developing cancer when exposed to a carcinogen."
Correct?
A Correct.
Q And you cite Park, Vitonis, and Wu for that. Is that correct?
A That's correct.
Q Looking at Park, isn't it true that Park does not supply any evidence to support your claim that mutations in BRCA1, BRCA2 and/or p53 increase a person's risk of developing cancer when exposed to a carcinogen?
A I'd have to remind myself of what's in

Page 339

Park.
Q Are you going through the entirety of the article?
A I'm just reminding myself the content to see if I could find something that was specifically related to your question about the presence of a BRCA1 or 2 mutation.
Q Okay. Is the BRCA1, BRCA2, p53, any of those even mentioned in the article?
And -- and I'm not sure we'll have time for you to go through each one of them in this much --
You've got -- you cited them for these propositions. I'm trying to ask you why you cited them for this proposition.
A I -- I'd have to look in more detail. I don't have a specific answer regarding the -- regarding BRCA1 --
Q Okay.
A -- I'm sorry -- BRCA genes.
I would suspect the Park reference was more in the discussion of overall relative risk of developing cancer and not necessarily exclusive to the presence of a mutation.

Page 340

So the -- the Park paper does discuss the relationship of ovarian cancer risk relative to benign gynecological conditions.
Q And -- and your comment that you've cited these studies for is the presence of these mutations increases a person's risk of developing cancer when exposed to a carcinogen. And these mutations would be what you've been talking about in this paragraph, the B -- the BRCA1, BRCA2, and p53; correct?
MS. O'DELL:
Object to the form.
A The sentence is worded, "The presence of these mutations increases a person's risk of developing cancer when exposed to a carcinogen."
MR. FERGUSON:
Q Right. Right.
And, for example, in Vitonis, isn't it true that BRCA1, BRCA2 and p53 were not even determined in that study and, instead, Jewish ethnicity was used as a surrogate for a woman's risk of having a mutation in one of these genes?
Do you recall that --
A Again, I would have --

Page 341

Q -- one way or the other?
MS. O'DELL:
Objection.
A I would have to review the -- review the paper. Because part of the review is to be -- include appropriate references with regards to ovarian cancer risk, and those may -- I think those publications provide some information in that space.
MR. FERGUSON:
Q All right. But when you cite studies for a statement in your report, shouldn't the studies relate to that statement?
MS. O'DELL:
Object to the form.
A Well, the studies relate to a person's risk of developing cancer. But I -- I think it -- it doesn't change the accuracy of the presence of the mutation relative to that risk. But the -- I don't have a -- a good answer as far as relationship of BRCA1 and 2 to the Park paper.
MR. FERGUSON:
Q And -- and, then --
Well, we talked about Vitonis, too.

Page 342

And then let's get to Wu.
MS. O'DELL:
Object to the form. You didn't comment specifically about Vitonis, if you've got an issue with Vitonis. You know, it's not fair to assume that because I don't think you asked a direct question.
MR. FERGUSON:
Okay. I thought I did, but I could be mistaken.
MS. O'DELL:
You mentioned it, but I don't think you -- I think it was more you rather than asking a question.
MR. FERGUSON:
Q With regard to Wu, do you recall that, in Wu, BRCA1, BRCA2, and p53 inherited carrier mutation status were not even determined in that study? Do you recall that --
A The --
Q -- one way or the other?
MS. O'DELL:
Object to the form.
A The Wu paper specifically discussed

Page 343

nongenetic risk factors.
MR. FERGUSON:
Q Let's go to the next paragraph, and there you talk about single nucleotide variance, SNVs; correct?
A Towards the bottom of the paragraph. As -- in terms of modifiers, yes.
Q Yeah. Are -- are single nucleotide variants mutations?
A Yes.
Q Do most SNVs result in functionally defective proteins?
A Statistically speaking on a genome-wide basis, no.
So a -- a single nucleotide variant is a variant at any point. And if we consider statistically that about 1 percent of the genome encodes proteins, again, it's statistically less likely that any SNV would affect a protein.
Q Okay. Let's look at the next paragraph. There you talk about Lynch syndrome; correct?
A Correct.
Q And you make a statement that Lynch

Page 344

syndrome patients have an increased risk of cancer when exposed to a carcinogen. Correct?
A Correct.
Q What carcinogens are you referring to?
A I'm not -- not referring to a specific carcinogen. I'm using the term "carcinogen" to refer to an insult that would result in DNA damage specifically because, similar to the BRCA mutations, Lynch syndrome impairs DNA mismatch repair.
So that defect alone is not sufficient to result in a cellular transformation, so something else has to occur. And when we consider that carcinogens are -- the term "carcinogen" generally refers to something that has the potential to damage cellular components or DNA, it's putting the --
Inability to repair along with the presence of a carcinogen is where that sentence comes from.
Q So -- and I want to make sure I understand what you're saying. Are you saying that Lynch syndrome patients have an increased risk of developing cancer after exposure to a

Page 345

carcinogen, just like everyone else?
A No. I'm stating that Lynch syndrome --
MS. O'DELL:
Object to the form. Excuse me.
A Lynch syndrome is a hereditary condition that increases the overall risk of cancer to an individual, similar to BRCA1 and 2 mutation.
MR. FERGUSON:
Q So you -- are you claiming that Lynch syndrome patients have a greater increase in relative risk when exposed to a particular carcinogen than do people without Lynch syndrome?
MS. O'DELL:
Object to the form.
A No, I'm not making that statement, to a specific carcinogen.
MR. FERGUSON:
Q In your next paragraph you talk of -- you start with "Myriad Genetics," and you say, "As with all inherited traits, a positive family history is the strongest indicator of the presence of genetic risk alleles in an individual."

Page 346

Correct?
A Correct.
Q Isn't it true that many women who have inherited mutations like BRCA1 or BRCA2 and genes that predispose to ovarian cancer development do not have a family history of breast or ovarian cancer?
A So the -- your -- your question is a little bit different than the statement. So the -- if I could clarify the statement in the report, it is more that a positive family history would be a likely indicator that someone has a genetic risk variant such as BRCA1 and 2.
Q Isn't it true that family history is not a sensitive or specific indicator of whether -- of whether a particular woman has inherited a mutation in a gene associated with increased risk of ovarian cancer?
MS. O'DELL:
Object to the form.
A I would say that family -- I would ask to define "sensitive" or "specific," because in genetics overall, family history remains a valuable and important characteristic in terms of

Page 347

determining the genetic component of -- of any disease, cancer included. And, so, if there's something exact regarding its sensitivity or specificity that I can comment on, I will if I know the answer. But...
MR. FERGUSON:
Q In -- in the top of the page -- of page 9, the next page, you indicate, "Because of the large number of individuals tested and the ability to trace their genetic inheritance, the genes involved in cancer development are well established."
Is that correct?
A Correct. That's what I state. I did make that statement.
Q And given that they're well established, can you name all of the inherited genes that have been identified as being associated with an increased risk of ovarian cancer?
A No, not -- I can't name them all off the top of my head, no. There's something in the neighborhood of 500 to -- 500 genes of strong association of cancer risk and progression, some

Page 348

number higher than that if you're looking at indirect or genetic complex formation.
You know, depends how far down the cellular control and signal transduction and growth and proliferation road that we go as far as how many genes. But I'm sure, as everyone well appreciates, everything in biology is interrelated in some form.
And, so, it -- but I would say this statement here is that our ability to look at large-scale genetic analysis in individuals of a variety of cancer types, given the number of individuals affected by cancer and the analysis of their genetics, we've been able to identify many of -- many of the fundamental or most -- perhaps most of the fundamental genes involved in that initial disease initiation or progression.
It's important that it is not a comprehensive list. Hence, it is not "all," but there are a large number of genes that are well established.
Q Okay. Let's look at the next page, 10. And you have a paragraph that starts "Macrophages."

Page 349

A Uh-huh.
Q And the last sentence says, "Generally speaking, macrophages can increase inflammation or decrease inflammation, depending on the cytokines released."
Correct?
A Correct.
Q So, with that statement, do you agree that inflammation can have both protumorigenic and antitumorigenic effects, depending on context, just as you state here for macrophages?
MS. O'DELL:
Object to the form.
A No, I -- I would not agree with that. I -- I don't know of any evidence of that, that inflammation, as a physiological phenomenon, acts as an antitumor effect.
MR. FERGUSON:
Q Going to the next page, the page 11 --
I'm trying to get through this hopefully within the next 15 minutes.
-- under the role of inflammation in ovarian cancer --
Are you with me there?

Page 350

A I am.
Q And you're obviously talking about the role of inflammation there. Isn't it true that no published animal model has ever shown that inducing inflammation induces the development of ovarian cancer?
MS. O'DELL:
Object to the form.
A We've been -- earlier today we were discussing some animal models as it relates to --
MR. FERGUSON:
Q Yeah. You and Miss Brown talked about a number of animal models.
A Yeah.
Q And -- and what I'm trying to ask you, is there any of those animal models or any others that have ever shown that inducing inflammation induces the development of ovarian cancer?
A I didn't -- I didn't look specifically for an animal study of that type in the process of developing the report.
Q Later down that page, you talk about two models. "The literature reviews as well as many direct studies feature the immune system as

Page 351

being an important mediator of ovarian carcinogenesis via two models, chronic inflammation and incessant ovulation."
Correct?
A Correct.
Q Is it your opinion that incessant ovulation is a form of chronic inflammation?
A It is not.
Q Isn't it true that there's no pathological evidence in humans that perineal talc users have ovarian inflammation?
MS. O'DELL:
Object to the form.
A I'm thinking.
I would have to review the --
I'm sorry. That's -- it's --
MR. FERGUSON:
Q Okay.
A I would -- again, I would have to look more carefully for that. I can't -- I can't name a study of that type right now.
Q So I think you've said previously --
Are you done looking?
I understood you couldn't give me

Page 352

anything on that, so that's -- that's fine.
Let's move on.
A Okay.
Q I think you've stated earlier that your opinion in this case is based on the totality of what is included in the product, the talcum powder products. Is that correct?
A Correct.
Q So you're -- you cannot distinguish the -- the carcinogenicity of the constituent parts of the talcum powder products, correct, including the fragrance?
MS. O'DELL:
Object to the form.
A I -- I was -- I was not asked to -- to provide that delineation. And, so, instead, subsequent to seeing some of the other expert reports, we began with talcum powder as a product and then have since learned more about the constituent components, including asbestos, fragrance, potential for heavy metals, which I understand or I've observed that there's a variety of testing documents that -- that show a variety of results.

Page 353

So, to answer your question, I did not specifically evaluate the individual specific components in any -- in any individual product as it relates. Instead, remained focused on the mechanism for the complete -- complete product.
MR. FERGUSON:
Q And you've made reference to heavy metals throughout your testimony on occasion. Do you recall that?
A I do.
Q Do you have any opinions that any of these heavy metals contribute to the inflammation process that you've been talking about?
A The -- to the inflammation --
I'm not aware of any direct evidence for heavy metal contribution to the inflammation process that we've been discussing. Instead, the heavy metals, particularly chromium, caught my attention because of its well-established ability to directly damage DNA and, therefore, you know, potentially play a role in carcinogenesis.
Q Do you have any knowledge or opinion about how much chromium you claim is in the -- in the body powder products?

Page 354

MS. O'DELL:
Object to the form.
A I wasn't asked to evaluate the amount of chromium or whether it was sufficient for damage. It was more reviewing. I would have to defer to other experts who have done the testing on the products.
MR. FERGUSON:
Q So you have no opinion on that?
MS. O'DELL:
Object to the form.
A I'm sorry. An opinion on the amount of chromium?
MR. FERGUSON:
Q Correct.
A Again, I wasn't asked to generate such an opinion.
Q I think -- I think I'm almost done.
Isn't it true that published data have demonstrated that talc is not genotoxic and does not cause mutations?
MS. O'DELL:
Object to the form.
A I'm not aware of a study that

Page 355

specifically looked at the genotoxicity of -- of talc. And I think it would certainly warrant defining which type of talc and components therein. But I'm -- I'm not aware of a study that has concluded that there are no genotoxic effects of any type of talc.
MR. FERGUSON:
Q Would you agree there's no evidence that talc causes sister chromatid exchange or unscheduled DNA synthesis?
MS. O'DELL:
Object to the form.
A I didn't -- I didn't review the literature for those two specific phenomenon. I would have to, again, specifically look or review for that.
MR. FERGUSON:
Q So, as you sit here, you have no opinion as to whether talc is or is not mutagenic?
MS. O'DELL:
Object to the form.
A No. We've -- so talc in general, particularly in its -- in its form of fibrous

Page 356

talc with asbestiform bodies, I think would be very reasonable to state that it has mutagenic properties.
MR. FERGUSON:
Q And can you cite me any literature for that?
A I would simply refer to the -- much of the body of asbestos literature for the -- for that.
MR. FERGUSON:
I think that's all I have. I'll turn it over to someone else to ask some questions.
MS. BROWN:
Anybody with some more?
MS. O'DELL:
I'm going to take a break for a few minutes.
VIDEOGRAPHER:
Going off the record. The time is 4:54 p m.
(OFF THE RECORD.)
VIDEOGRAPHER:
We're back on the record. The time is 5:20 p m.

Page 357

EXAMINATION
BY MS. O'DELL:
Q Dr. Levy, I have just a few follow-up questions for you.
I'm gonna ask you to turn to page 14 of your report.
And earlier today --
I'm going to ask, Doctor, if you could put the exhibits in front of you, and we'll pull those out.
But earlier today you were asked about a letter from the FDA that was marked as Exhibit Number 16, and if you could pull that out of your stack there. And, specifically, if you'll turn to page 4 of the letter.
And you'll recall that this letter was written in 2014. Do you remember that?
A Yes.
Q And if you look, however, at page 4 of the letter, it appears that the FDA's review of the relevant toxicity literature stopped at the year 2008. Fair?
MS. BROWN:
Objection to the form.

Page 358

MS. O'DELL:
Q Did the FDA's review of the toxicity literature stop in 2008?
A Yes.
Q And if you look at page 14 of -- of your report, your review of the literature included multiple references that were published after 2008?
MS. BROWN:
Form.
A That's correct.
MS. O'DELL:
Q And, in fact, you cited Shukla that was published in --
Was Shukla published in 2009?
A Yes. The reference is in the report to 2009.
Q Yes.
And, in addition to that, did you cite other references in support of your opinion that talc powder causes inflammation that were dated and published after 2008?
A I did.
Q And, so, the suggestion by counsel for

Page 359

Johnson & Johnson that somehow the FDA had reviewed the literature for toxicity up until the date of this letter would have been incorrect?
MS. BROWN:
Objection to the form of the question.
A As -- as we discussed, the -- the letter from the FDA dated April 1st, 2014, states to include literature from 1980 to 2008.
MS. O'DELL:
Q Let me ask you --
You can put that aside, Dr. Levy. Thank you.
And I want to ask you to pull out of the stack the Exhibit 17, which is the Buz'Zard paper.
A I have it.
Q And if you'll turn to page 581.
A Okay.
Q And just to orient our discussion, counsel for Johnson & Johnson suggested that -- that this paper showed a decrease in reaction or reactive oxygen species at the longest time interval. Do you recall that discussion?
MS. BROWN:

Page 360

Objection to the form of the question.
A Yes, we -- we had a discussion regarding the results shown in Figure 3, the level of exposure of talc as well as its duration. Sorry. The talc dose as well as duration.
MS. O'DELL:
Q And in the -- if you'll look at Figure 1, Doctor, explain to us, please, what Figure 1 describes in terms of the viability of the cells at the 72-hour mark.
A So the -- so Figure 1 is a graph describing percent cell viability versus the different normal or variant cells at a 24-hour and 72-hour time point, two different ovarian cancer cell lines, as well as doses of talc from zero micrograms per milliliter up to 500 micrograms per milliliter, and each of those is applied.
And at the 72-hour time point in both cell lines, OSE2a and GCA1 -- GC1a shows a decrease in cellular viability that is dose-dependent in each of the four cell lines.
Q Okay. And --

Page 361

A Sorry. Each of the two cell lines.
Q And is it fair to say that the reason you don't see dose response, you know, at the -- at the greatest magnitude is because the cells essentially die?
MS. BROWN:
Objection to the form.
A Well, I would say if we consider the results displayed in Figure 1 in relation to the results displayed in Figure 3, an ex- -- an explanation for the concentrating on the 500 -- the highest dose, the 500 micrograms per milliliter, in the talc exposure, the decrease in cellular viability is an -- is an explanation -- could be an explanation for the decrease in reactive oxygen species.
MS. O'DELL:
Q Okay. Thank you, Doctor.
And if you'll put that aside and turn to Exhibit 7, which was the Hamilton paper we spent quite a lot of time on earlier.
Do you recall the -- that discussion regarding the Hamilton paper?
A I do.

Page 362

Q And what was the purpose for which you cited the Hamilton paper?
A That it was one of the available animal studies looking at the effects of talc on a rat ovary.
Q And did the paper show that there was a increase in inflammation as result of talc?
A Yes, in the form of foreign body granulomas observed in five of the injected ovaries.
Q And you're looking at, I guess, that last sentence on page 103 and carrying over to the -- to the narrative on page 105?
A Cellular foreign body?
Q Yes.
A Foreign body granulomas without any surrounding inflammation were seen in five of the injected ovaries. And similar lesions were not uncommonly noted in the supracapsular fat in the connective tissue matrix of the capsule.
Q And if you'll look down in the discussion section, Dr. Levy, the first paragraph there in your -- where -- beginning "Unfortunately," does it appear that talc also

Page 363

induced fibrosis --
MS. BROWN:
Objection to form.
MS. O'DELL:
Q -- in the rats?
A The manuscript makes the statement that, "Unfortunately, bursal distention occurred as an unforeseen complication" and further states that this probably resulted from talc-induced fibrosis and obliteration of the small channel which normally allows communication between the cavity where the ovary lies and the perineum.
Q And though the authors concluded that neoplastic changes were not seen, the authors did find evidence of inflammation in their study?
A That's correct.
Q Prior to becoming involved in the litigation, Dr. Levy, did you hold the opinion that inflammation is a cause of cancer?
A As -- as we've discussed earlier, I certainly held the opinion that, you know, inflammation is a significant and necessary component of cancer progression.
Q And has that been -- that general

Page 364

principle been published in the peer-reviewed literature?
A It has.
Q And, in regard to ovarian cancer, prior to becoming involved in the litigation, did you hold the opinion that inflammation was a part of the development of ovarian cancer?
A Yes.
Q And has that been researched and that research published in the peer-reviewed literature?
A It has.
Q In the same way, has the fact that talc, talcum powder, induces inflammation been published in the peer-reviewed literature?
MS. BROWN:
Objection to the form.
A Yes.
MS. O'DELL:
Q And you were asked whether there was evidence that talc caused inflammation in humans. Do you recall that question?
A I do.
Q And based on your exhaustive review of

Page 365

the literature, what evidence would you point to undergirding your opinion that talc causes inflammation in humans?
A I think considering the molecular mechanism we were discussing of the recent paper by Saed, et al., again, that we discussed earlier today is a fairly in-depth set of experiments to examine the specific inflammatory response of -- of human cells to -- to talcum powder.
Q In addition to the Saed publications, would you -- would you include the Shukla 2009 paper in your consideration of talc causing inflammation in humans?
A Yes.
MS. BROWN:
Form.
MS. O'DELL:
Q You were asked about your methodology numerous times today, and can -- would you describe in -- in general the methodology you have used in reaching your opinions in this case?
A Yes. To clarify or perhaps expand on the earlier discussions, my methodology involved a literature review to examine the totality of

Page 366

the information available to the role that talcum powder plays in inflammation in ovarian cancer.
And, so, that methodology involved, first, a review of the literature and then a development of a report and then a synthesis of a biologically plausible mechanism where the basis of that plausibility was to ask if each of the different component steps that are described in that mechanism was supported by peer-reviewed research. First, does talc cause inflammation? Second, does inflammation cause cancer? And then, third -- or does inflammation cause ovarian cancer? And then, third, is there -- is that supportive of a overall mechanism of cancer progression and metastasis?
Q Can that methodology be replicated?
A Certainly. I think, you know, anyone with a similar -- similar background and experience who -- who undertook the same activities would likely -- certainly likely come up with the same -- same conclusions.
Q Did you rely on the IARC monograph in relation to nickel, chromium, and cobalt in reaching your opinions in this case?

Page 367

MS. BROWN:
Objection to the form.
A I -- so the -- the number of IARC publications were certainly in the material that was reviewed for -- for my -- for my report.
MS. O'DELL:
Q Based on your review of the literature, is it your opinion that nickel causes inflammation?
A Yes. The IARC -- the -- the characterization of those compounds, nickel as well as chromium, among others, are -- would have an inflammatory response.
Q You were asked questions earlier today -- actually, not so much earlier -- a few minutes ago regarding the Park paper. And you cited the Park paper on page -- I think it was 8 of your report.
A Yes.
Q And let me show you what I'm marking as Exhibit 22 to your deposition.
(DEPOSITION EXHIBIT NUMBER 22 WAS MARKED FOR IDENTIFICATION.)
MS. O'DELL:

Page 368

Q Is this the Park paper that you referenced --
MS. BROWN:
Counsel, do you have a copy for us?
MS. O'DELL:
I don't. I'm assuming -- I don't think Ken marked it, but I'm assuming he has a copy.
Q Is that the Park paper that you referenced in your report, Dr. Levy?
A It is.
Q And if you'll turn to page 8 of the paper, about midway down the first column, maybe a little bit less, see the paragraph starting "We did find an association"? Page 8.
A I'm looking for the page number.
Q Sorry. Let me give you a page number. I'm not sure it has a page number.
A No, it doesn't.
Q Do you see the paragraph beginning "We did find associations between overall cancer and history of fibroid or ovarian cysts"? Do you see that paragraph?
A Well, actually -- yes, I see that paragraph.

Page 369

Q If you'll look further, the sentence beginning "This observation may suggest," do you see that?
A Yes. Uh-huh.
Q And the paper says, "This observation may suggest a possible additive or synergistic effect on tumor- -- tumorigenesis influenced by the proinflammatory milieu from an increased burden in the number of benign conditions. Increased risk of serous cancer, ovarian cancer, women with other proinflammatory risk factors has been reported -- reported, most notably in talc users."
Do you see that?
A I do.
Q Is that the section you were thinking of when you cited it in your report?
MS. BROWN:
Objection to the form.
A Yes, it is.
MS. O'DELL:
Q Let me ask you to -- a couple of other final questions, Dr. Levy.
Excuse me. Give me one moment.

Page 370

In regard to opinions in relation to the pathology of ovarian tissue, would you defer to a gynecologist or gynecologic oncologist or a pathologist regarding that matter?
A Yes, of course.
Q You testified earlier today that you relied on the Longo testing in -- in reaching your opinions in this case.
MS. BROWN:
Objection to the form.
MS. O'DELL:
Q Did you rely on Dr. Longo's testing in -- in reaching your opinions in this case?
A Yes. They were -- they were one of the -- among many of the manuscripts we've been discussing.
Q Yeah.
In fact, you cite Dr. Longo's report on page 15 of your report. Is that right?
MS. BROWN:
Objection to the form.
A Yes.
MS. O'DELL:
Q And -- and in terms of Dr. Longo's

Page 371

report, his findings of 37 of 56 historical talc samples being positive for asbestos and 41 of the 42 samples tested containing fibrous talc, was -- was that information you had prior to reaching your opinions and finalizing your report?
MS. BROWN:
Objection to the form.
A Yes.
MS. O'DELL:
Q And in relation to Dr. Crowley's report regarding the fragrance chemicals, do you defer to Dr. Crowley regarding his analysis of the fragrance chemicals?
A Yes.
Q And did you rely on the opinions he reached in relation to the fragrance chemicals in reaching your opinions in this case?
A Yes. My -- my review of that just, in addition to deferring it, was -- just made the general -- or made the statement that I was in general agreement with his opinions in those matters, seeing as that's not a -- not an area of expertise of mine.

Page 372

Q And did you have the opportunity to consider his report prior to finalizing your report?
A I did.
Q I have nothing further. Thank you.
EXAMINATION
BY MS. BROWN:
Q Dr. Levy, would you take Exhibit 16 out, please, the FDA's response to the citizens petition?
A I have it.
Q Counsel asked you some questions that involved questions that I asked you. Remember she asked you the lawyer for J & J didn't point out the articles that were reviewed from 1980 to 2008 on page 4? Do you recall those questions from plaintiffs' counsel?
A Yes.
Q Would you look at the last page of the letter, page 6 of 7? I'd like to direct your attention to the second sentence on this page that begins "In consideration of your request." Do you see that?
A I do.

Page 373

Q And it states, "In consideration of your request, we conducted an expanded literature search dating from the filing of the petition in 2008 through January 2014. The results of this search failed to identify any new compelling literature data or new scientific data."
Do you see that?
A I see that.
Q And putting together, then, the information from page 4 and page 6, you see that the FDA considered literature from 1980 to 2014. Is that correct?
MS. O'DELL:
Object to the form.
A Yes, that is correct.
MS. BROWN:
Q And what the FDA concluded, contrary to your opinion here, Doctor, is that a cogent biological mechanism by which talc might lead to ovarian cancer is lacking; correct?
MS. O'DELL:
Object to the form.
A That's in this --
MS. BROWN:

Page 374

Q Directing your attention to page 4, number 4, the conclusion regarding a cogent biological mechanism lacking. Do you see that?
MS. O'DELL:
Object to the form.
A Yes. I see where they -- they made the statement that cogent biological mechanism by which talc might lead to ovarian cancer is lacking and that exposure to talc does not account for all cases of ovarian cancer.
MS. BROWN:
Q Next, Doctor, do you rely on the findings of the Hamilton article in forming your opinions in this case?
A Similar to as we've discussed, in a portion, yes.
Q You, Dr. Levy, cannot point us to a single paper showing an inflammatory response leading to ovarian cancer in humans from talc use. True?
A There is -- I do not know of a single paper that -- in a controlled fashion in humans provided talc exposure that then was -- subsequently led to cancer in humans. That's

Page 375

correct.
Q Controlled aside, you're not aware of any observational case report, any kind of study that shows talcum powder use causing an inflammatory response leading to cancer in humans; correct?
MS. O'DELL:
Object to the form.
A I would -- my review and development of the biological plausibly -- plausible mechanism examined literature that led to the conclusions described in the report. I'm not aware of a --
The human-based studies were all case cohort and -- or case-controlled and cohort studies that showed an association with talc exposure and cancer, but I'm not aware of a direct study.
MS. BROWN:
Q There have been some reports of alleged findings of talc in tissues or in other parts of the body. Are you familiar with those?
A Yes.
Q And you're not aware of any one of them demonstrating an inflammatory response that the

Page 376

talc was causing in the body. True?
MS. O'DELL:
Object to the form.
A I'm aware of a number of studies that looked at inflammatory response in model systems and cell lines, and additional studies that looked at inflammation in humans I believe were referenced.
Certainly the Penninkilampi manuscripts described inflammatory observations and -- as well as the Buz'Zard and Lau were on human cells.
Q Dr. Levy, is it your testimony that the Penninkilampi meta-analysis of prior case-controlled studies demonstrated a inflammatory response of -- from perineal use of talc that led to ovarian cancer?
MS. O'DELL:
Object to the form.
A No. That's not my statement. It was that those -- those papers reported an inflammatory observation as part of those studies.
MS. BROWN:
Q Not in the tissue from talc; right,

Page 377

Doctor?
MS. O'DELL:
Object to the form.
A It would be those studies in the meta review were not examining the tissue content for talc. So they're unable to make that determination.
MS. BROWN:
Q So we must be missing. I'm -- what I'm asking you is for any study at all in the whole world that shows that talcum powder in somebody's body causing an inflammatory response that led to ovarian cancer. Can you name one?
MS. O'DELL:
Object to the form.
A I mean, we've -- we've discussed a number of studies that described the risk and association of talc in ovarian cancer. But the limitation of the -- of your question or the limitation of the studies relative to your question is those particular studies may not have also assessed the inflammatory response or an inflammatory response, given the nature of the studies.

Page 378

MS. BROWN:
Q Well, we got one. We got the Heller study that purported to find talc in ovarian tissue; right?
MS. O'DELL:
Object to the form. Different --
MS. BROWN:
Counsel, it's form, please.
MS. O'DELL:
Object to the form.
A Yeah. What was the -- the Heller study, here it is.
Yes, I recall our discussion of this paper.
MS. BROWN:
Q Right.
And this study reported that there was no inflammatory response around the talc that they claimed to have found in the ovarian tissue. True?
A They make those statements in the paper, but the -- the -- I would have some concern with the histological methods, but I would certainly defer to a pathologist in the

Page 379

sense of being able to determine the both presence of talc and the inflammatory response in that.
Q So you have some critiques of the Heller study. Is that fair?
MS. O'DELL:
Object to the form.
A I would say I would need a -- I would need a -- a -- I would need a further evaluation of the methodology for detecting both talc as well as inflammation in the same materials using the methods of the Heller paper.
MS. BROWN:
Q Are you aware of any other paper that you think is methodologically superior that shows the presence of talc in ovarian tissue exhibiting an inflammatory response?
MS. O'DELL:
Object to the form.
A Well, we've discussed the rat studies.
MS. BROWN:
Q Human tissue. That's my question.
A Human --
Q Human tissue.

Page 380

MS. O'DELL:
Actually, that wasn't your question. But you've clarified it, so --
A The -- so you're excluding -- are you excluding cell lines?
MS. BROWN:
Q Yeah. Human beings. Do you know of any study like Heller in human beings that purports to find talc in human women ovarian tissue that shows an inflammatory response?
MS. O'DELL:
Objection to form.
A I'm not aware of a study showing that specifically.
MS. BROWN:
Q Counsel asked you some questions about nickel causing inflammation that leads to ovarian cancer. Do you recall those?
MS. O'DELL:
Object to the form.
A No. I was asked if -- if heavy metal -- or components like nickel have been shown to have a potential inflammatory response.
MS. BROWN:

Page 381

Q Uh-huh. Because you're not aware of any published scientific literature that shows heavy metals cause inflamma- -- inflammation that leads to ovarian cancer; right?
A I wasn't asked to -- to review for that. I would state that there's a number of studies that show the role of metals -- particularly chromium -- and its -- and its damaging effect on DNA, which I think by -- would certainly have both an inflammatory as well as carcinogenic effect.
Q And we're here on an issue of ovarian cancer. And, as it relates to ovarian cancer, you're not aware of any scientific support for the proposition that heavy metals can lead to inflammation that causes ovarian cancer. Fair enough?
A Well, I was -- certainly, I was asked to review the literature to develop a -- and develop conclusions of that literature as it related to a -- a potential or possible biological mechanism.
In doing that, in part of that review, we certainly made the observation that talc and

Page 382

its components, as we discussed earlier, may have -- there's the possibility of having additional component effects, such as heavy metals and their effects, asbestiforms and their effects and the like; therefore, really considering the complete components of the product overall.
Q And, as it relates to the testimony you just gave, you're talking about just a theoretical possibility; right?
MS. O'DELL:
Objection to form.
A Sure. And, then, from that review developing a -- a conclusion of a biologically plausible mechanism.
MS. BROWN:
Q Has that conclusion been published in the peer-reviewed literature, Doctor?
A No, it has not.
Q And, in fact, as you -- all of the opinions that you gave here today, those opinions have not been published in the peer review literature. True?
MS. O'DELL:

Page 383

Object to the form.
A Not at this time.
Q Counsel asked you some questions about Dr. Longo. Do you recall that?
A Yes.
Q You've done nothing to validate the findings that Dr. Longo writes about in his reports. Is that fair?
A No, I have not done any experiments to validate those findings.
Q Okay. Are you aware that some of the samples that Dr. Longo tests and purports to find asbestos were purchased off of eBay?
MS. O'DELL:
Misstates -- well --
A My review of the report, I was -- did not include the -- I guess the specific history of each of the samples.
MS. BROWN:
Q Do you understand that asbestos -- that minerals like tremolite or anthophyllite, they exist in both the asbestiform and nonasbestiform way?
A I would defer to other experts on the

Page 384

mineralogy of talc.
Q And whether what Dr. Longo is finding in the samples that he tested is the asbestiform or nonasbestiform variety of the minerals, you would defer to others? Is that fair?
A I'd certainly defer to Dr. Longo.
Q And have you looked at any other testing of the samples that Dr. Longo has tested?
MS. O'DELL:
Object to the form. Vague.
A Within the literature, there's -- there was a number of tables describing testing, described tests from previous testimony.
MS. BROWN:
Q Have you looked at the testing that public health authorities like the FDA have done on Johnson & Johnson's baby powder?
A I believe some of that was provided, yes.
Q Are you relying on any finding of asbestos from Dr. Longo in forming your opinions here today?
A The --
MS. O'DELL:

Page 385

Object to the form.
A The inclusion of the asbestos, again, as -- as -- as we've discussed a few times today, the conclusion I developed from the report were not dependent or independent of any one or another component of -- of the talcum powder.
As we discussed a bit ago, the presence of asbestos as a known inflammatory mediator, as well as potential carcinogen, I think just helps lend additional support to the biological plausibility of the mechanism. But I think that biological mechanism is not dependent on the presence of asbestos.
MS. BROWN:
Q Other than plaintiffs' expert, Dr. Longo, are you relying on anything else to support the potential for asbestos in baby powder?
MS. O'DELL:
Object to the form.
A There's -- so I saw reference to asbestos content in some of the other literature that was reviewed during the time, and, so, there were other publications that made mention of the

Page 386

asbestos content in talc during the overall review.
MS. BROWN:
Q Sitting here today, are you aware whether or not that was Johnson & Johnson's cosmetic talc?
MS. O'DELL:
Object to the form.
A I would have to look closely. I'm not aware of that specifically.
MS. BROWN:
Q Counsel asked you some questions about Dr. Crowley and whether or not you were relying on the opinions he reached. Do you remember those questions?
A I do.
Q What opinions did Dr. Crowley reach on which you rely?
A Dr. Crowley performed an analysis of the fragrance components and made assessments of the individual chemical components and their relationship to -- or I should say their -- their inclusion on various lists for their -- their chemical properties or safety. And in most -- in

Page 387

the majority of cases, the chemicals were not listed. In a number of cases, there were large numbers of chemicals listed as either irritants and, therefore, able to cause inflammation, or, in a few cases, as potential carcinogens.
And, so, it was that review of that information, similar to our discussions around asbestos, that I included or agreed with his opinions regarding that on the last paragraph or close to the last paragraph of the report that stated I was just in agreement that these -- that those chemicals contribute to the inflammatory properties observed.
Q Do you know in what quantity the chemicals Dr. Crowley identifies are present, if at all, in Johnson & Johnson's products?
A No. I wasn't asked to provide that review. I would defer to Dr. Crowley's report regarding any quantitative analysis of those chemicals.
Q And, as it relates to your opinion, Dr. Levy, it makes no difference whether Dr. Crowley's list has ten chemicals in Quantity X or five chemicals in Quantity Y. Your

Page 388

opinion is independent of Dr. Crowley's findings. Is that fair?
MS. O'DELL:
Objection to form. Vague.
A Well, my -- my -- my opinion, again, similar to -- as we've been discussing that, it considers the totality of the information available, including Dr. Crowley's report, but does not rely on any one specific report or otherwise.
And, so, the -- again, restating similar to the asbestos, the presence of potential irritants as another component in the -- in the product just provides additional support for that inflammatory mechanism playing a significant role.
MS. BROWN:
Q If none of the chemicals Dr. Crowley identified were present in baby powder, would you hold the same opinion of biological plausibility?
A I would.
Q If no asbestos was present in baby powder, would you hold the same opinion on biological plausibility?

Page 389

A Yes.
MS. BROWN:
No further questions. Thank you.
MS. O'DELL:
I have just one follow-up.
Or do you have anything --
MR. FERGUSON:
Nothing further.
MS. O'DELL:
Excuse me. I'm sorry.
EXAMINATION
BY MS. O'DELL:
Q Dr. Crowley, are your opinions in this case contained in your report as well as in the testimony that you've given here today?
A You said Dr. Crowley.
Q Oh. Excuse me. Sorry. I had Dr. Crowley on my mind.
Dr. Levy --
It's getting late in the day.
Dr. Levy, are your opinions in this case expressed in your report and your testimony today?
A Yes.

Page 390

Q And do you hold those opinions to a reasonable degree of scientific certainty?
A Yes.
MS. O'DELL:
I have nothing further.
MS. BROWN:
Thanks for your time, Doctor.
I think we're off the record.
VIDEOGRAPHER:
We're off the record. The time is 6 p.m.
(Deposition concluded at 6:00 p.m.)

Page 391

CERTIFICATE

I do hereby certify that the above and foregoing transcript of proceedings in the matter aforementioned was taken down by me in machine shorthand, and the questions and answers thereto were reduced to writing under my personal supervision, and that the foregoing represents a true and correct transcript of the proceedings given by said witness upon said hearing.

I further certify that I am neither of counsel nor of kin to the parties to the action, nor am I in anywise interested in the result of said cause.

LOIS ANNE ROBINSON, RPR, RMR
REGISTERED DIPLOMATE REPORTER
CERTIFIED REALTIME REPORTER

Page 392

ERRATA PAGE

I, SHAWN LEVY, Ph.D., the witness herein, have read the transcript of my testimony, and the same is true and correct, to the best of my knowledge, with the exceptions of the following changes noted below, if any:

Page/Line	Word(s) to be changed/reason	Correct Word
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SHAWN LEVY, Ph.D.

Page 393

DECLARATION OF WITNESS

I, the undersigned, declare under penalty of perjury that I have read the foregoing transcript, and I have made any corrections, additions, or deletions that I was desirous of making; that the foregoing is a true and correct transcript of my testimony contained herein.

EXECUTED this _____ day of _____, 2019, at _____, _____.
(City) (State)

_____
SHAWN LEVY, Ph.D.

J